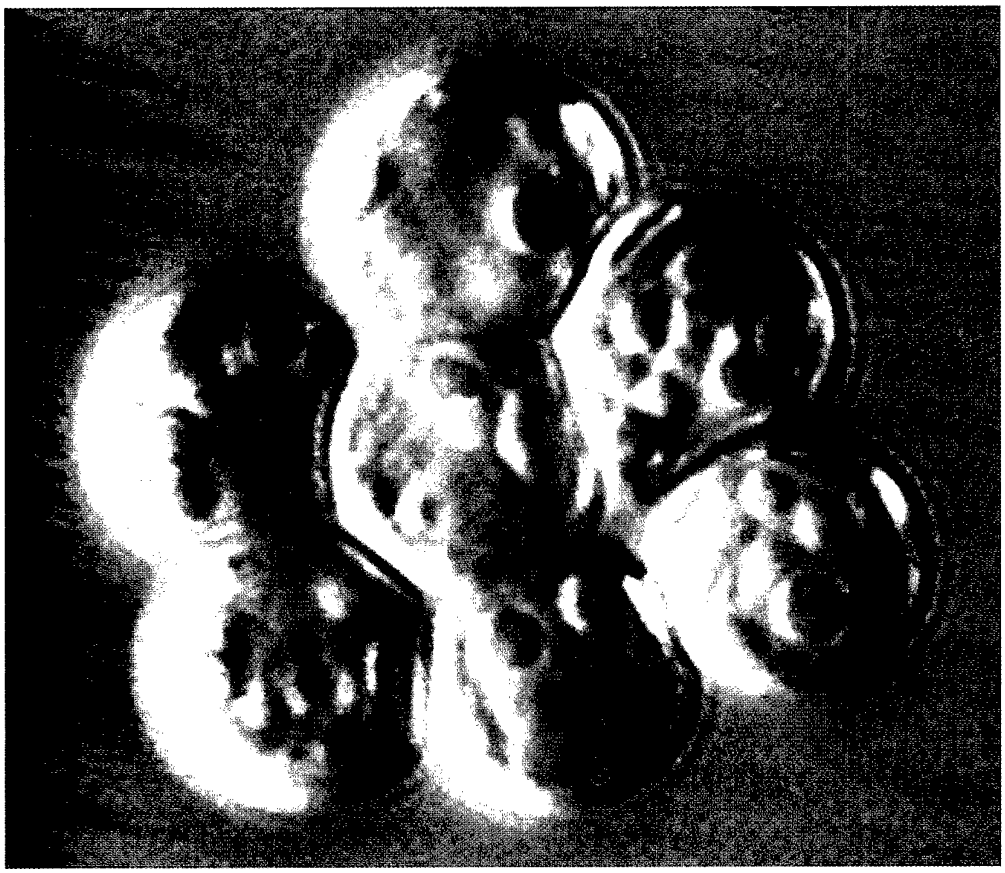
Marine Biofouling

An International Symposium sponsored by the Natural Environment Research Council, the Office of Naval Research and the Office of Naval Research, Europe



Programme and Abstracts

7-9 July 1999

Natural Environment Research Council

Marine Biofouling

An International Symposium sponsored by

Natural Environment Research Council
Office of Naval Research
Office of Naval Research, Europe

at the
Robbins Conference Centre
University of Plymouth

Student bursaries provided by the
Marine Biological Association

Cover: *Enteromorpha* spores
courtesy of Professor J.A. Callow

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MARINE BIOFOULING

Robbins Conference Centre
University of Plymouth

Tuesday, 6 July

16.00 – 18.00	Registration; set up posters (continues at 08.00, 7 July)
19.00	Welcoming reception

Wednesday, 7 July

08.30	Opening remarks
09.00	Biofouling and antifouling research in tropical systems: Indian Ocean studies and comparisons – A.B. Wagh & B.J. Zahuranec

SESSION 1 – SURFACE CHARACTERISATION AND CORROSION
Chairperson – L.V. Evans

09.20	**Keynote address:** Microscopies, spectroscopies and spectrometries applied to marine corrosion – B. Little
10.00	The role of bacterial exopolymers in marine fouling and deterioration of marine surfaces – R. Gubner, V. Zinkevich, L. Hanjangsit, I. Beech & R. Avci
10.20	The use of infrared spectroscopy as a probe for monitoring the metal/biofilm/solution interfaces – J. Halsall, M. Kalaji & A. Neal
10.40	**COFFEE**
11.10	**Keynote address:** Characterization of a model conditioning film – P. Suci
11.50	Structural and chemical characerisation of echinoderm non-fouling surfaces – M.M. Grundy, D. Giantzoudis, C.D. Bavington, N.V. Richardson & J.D. McKenzie
12.10	Studies of the cross-linking mechanism of mussel adhesive proteins and barnacle cement – K. Mjörn, C. Fant, F. Höök & H.Elwing
12.30	**LUNCH**

SESSION 2 - MICROFOULING
Chairperson – W.A. Hamilton

14.00	**Keynote address:** The molecular and ecological diversity of bacterial adhesion to surfaces – M. Fletcher
14.40	The control of biofilms by signaling molecules and signal analogues – J.W. Costerton

15.00	Interactions between microbial biofilms and attachment of *Enteromorpha* spores – I. Joint, M.E. Callow & J.A. Callow
15.20	**Keynote address:** Bioadhesion and bioadhesives in marine raphid diatoms – R. Wetherbee, M. Higgins, S. Crawford, N. Poulsen, A. Bacic & P. Mulvaney
16.00	**TEA**
16.30	Interplay of surface chemistry, colonization promoters, diatom-diatom and diatom-bacterial interactions in marine biofilm formation – K.E. Cooksey, & B. Wigglesworth-Cooksey
16.50	Predation by protozoa on bacteria deposited on surfaces – M.A. Sleigh & M.V. Zubkov
18.30 – 22.30	**BOAT TRIP (from the Barbican)**

Thursday, 8 July

SESSION 3 – MACROFOULING
Chairperson – G.W. Swain

08.40	**Keynote address:** Substratum/bacterial interactions and larval settlement – J. Maki
09.20	A field analysis of settlement of *Semibalanus balanoides* cyprids in response to substratum biofilming: the effects of diatoms, bacteria and grazers – C.D. Todd, H. Gurney-Smith & A.S. Clare
09.40	Barnacle settlement: field experiments on the influence of larval supply, tidal level, biofilm quality and age of *Balanus amphitrite* (Darwin) cyprids – F. Olivier, R. Tremblay, E. Bourget & D. Rittschof
10.00	Larval settlement and metamorphosis in the serpulid polychaete *Hydroides elegans* (Haswell) in response to cues from bacterial films – S.K. Lau & P.-Y. Qian
10.20	The effect of air drying a biofilmed surface upon larval settlement preferences of the tubeworm *Pomatoceros lamarkii* (Polychaeta: Serpulidae) – J.P. Hamer & G. Walker
10.40	**COFFEE**
11.10	Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitory cues for larval settlement in the polychaete *Hydroides elegans* – T. Harder & P.Y. Qian
11.30	Understanding settlement and primary adhesion in *Enteromorpha* – J.A. Callow, M.S. Stanley & M.E. Callow
11.50	Towards an understanding of gregariousness in barnacles – K. Matsumura, Y. Yamazaki, H. Munasinghe, M. Ogiso & A.S. Clare

12.10 Prostanoids and their effects on larval settlement in the barnacle, *Balanus amphitrite* – A.F. Rowley, J. Knight, A.S. Clare

12.30 **LUNCH**

Chairperson – P.D. Steinberg

14.00 Are differences in settlement generated by variation in the exploratory behaviour of cyprids? – J.M. Hills, J.C. Thomason, A. Cook, H.M. Davis, E.K. Millet, F.G. Pannacciulli, G. Relini, S. Sandrock, E.-M. Scharf & G.W. Swain

14.20 Surface texture within a narrow range eliminates settlement of the barnacle *Balanus improvisus* – K.M. Berntsson, P.R. Jonsson & H. Andreasson

14.40 Attraction and deterrence: settlement behaviour bioassays for the screening of non-toxic coatings – J.C. Thomason, J.M. Hills, P.O. Thomason, A.S. Clare & K. Matsumura

15.00 **POSTER SESSION**

(1) Chemical cues for *Entromorpha* settlement – A. Jennings, J.A. Callow & M.E. Callow

(2) The effect of cypris age on barnacle settlement – K. Matsumura, M. Yamazaki, P.A. Smith & A.S. Clare

(3) Supply and settlement dynamics of *Semibalanus balanoides* cyprids: the weak link in the chain? – J.C. Thomason, J.M. Hills, S. Wieczorek, C.D. Todd, R. Head, S. Hornby, K. Last & J. Warren

(4) The timing of field settlement assays: the consequences of small differences in deployment date – J.C. Thomason, J.M. Hills & P. Mapson

(5) The effect of hydrodynamics on the three-dimensional structure of barnacle colonies - J.M. Hills, J.C. Thomason, S.E. Hornby & A. Dolan

(6) Settlement behaviour of barnacle larvae in response to wood treated with a leaching-resistant anti-borer treatment containing copper, chromium and arsenic (CCA) – F, Fernandez-Estarlich, S.M. Cragg & T.G. Nevell

(7) Isolated fractions from the marine sponge *Geodia barretti* with inhibition of settlement in the barnacle *Balanus improvisus* – M. Sjögren, U. Göransson, P. Jonsson, P. Claeson & L. Bohlin

(8) The antifouling benefits of a scallop/sponge symbiosis – J.C. Thomason, J.M. Hills, A. Neville & S. Wieczorek

(9) Non-toxic defence against fouling with natural and synthetic repellents – S.V. Dobretsov & A.I Railkin

(10) Electrochemical method of protection against marine growth adhering to ship hulls – S. Sandrock & E.-M. Scharf

(11) Biofouling of optical instruments: The problem and potential solution – R.M. Head, J.C. Thomason, R. Menlove, & J.D. Davenport

16.00 **TEA**

16.30	An overview of the key achievements of the Fusetani Biofouling Project – H. Hirota & N. Fusetani
16.50	Ship hull fouling as vector of species introductions – S. Gollasch
17.10	The effects of marinas on the distribution of macro-fouling organisms around Melbourne, Australia: observation, experimentation and speculation – J.A. Webb, M.J. Keough & A.J. Butler
19.30 for 20.00	**SYMPOSIUM DINNER (Babbage Building)**

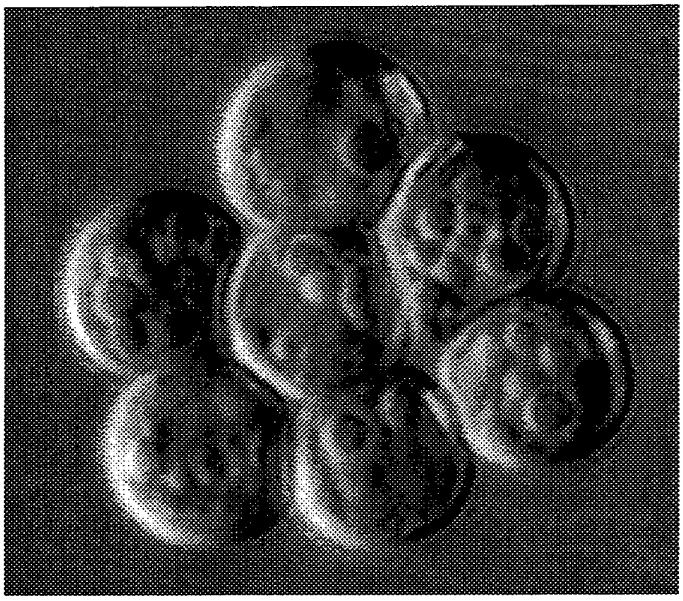
Friday, 9 July

SESSION 4 – TOWARDS ALTERNATIVE TECHNOLOGIES TO METAL-BASED ANTIFOULING COATINGS
Chairperson – B. Little

08.40	**Keynote address:** Towards non-toxic fouling-resistant coatings: Design and preparation of low-surface energy coatings with controlled surface chemistry and microstructure – P. Gatenholm, M. Andersson, M. Berglin, M. Jelvestam & K. Wynne
09.20	Nontoxic fouling release; correlation of polymer surface properties with ease of fouling removal – K.J. Wynne, S. Bullock, J. Uilk & R.B. Fox
09.40	The performance of fouling-release coatings: Static immersion at seven sites worldwide – G. Swain, A.C. Anil, R.E. Baier, E. Conte, A. Cook, M. Hadfield, E.G. Haslbeck, E. Holm, C. Kavanagh, D. Kohrs, C. Lee, L.Mazzella, A.E. Mayer, P.-Y. Qian, S.S. Sawant, M. Schultz, J. Sigurdsson, C. Smith, L. Soo, A. Terlizzi, A.B. Wagh, R. Zimmerman & V. Zupo
10.00	Temporal and spatial variations of macro-fouling on silicone biofouling release coatings – C. Darkangelo Wood, K. Truby, J. Stein, D. Wiebe, E. Holm, D. Wendt, C. Smith, C. Kavanagh & A. Meyer
10.20	The surface properties of some silicone and fluorosilicone coating materials immersed in seawater – F. Fernández Estarlich, S.A. Lewey, T.G. Nevell, A.A. Thorpe, J. Tsibouklis & A.C. Upton
10.40	**COFFEE**
11.10	The roles of surface energy and frictional processes in release phenomena – M.K. Chaudhury & K. Vorvolakos
11.30	Preventing the bacterial colonisation of surfaces: the non-stick-coating approach – P. Graham, I. Joint, T. Nevell & J. Tsibouklis
11.50	Mechanism of barnacle removal from silicone coatings – I.L. Singer & J.G. Kohl

12.10	Surface active neurotransmitter antagonists prevent the settlement of cyprid larvae – M. Dahlström, L. Mårtensson, P. Jonsson, M. Wallin & H. Elwing
12.30	**LUNCH**
	Chairperson – C.D. Todd
14.00	**Keynote address:** Chemical ecology of surface-based interactions – P.D. Steinberg
14.40	Antifouling activity of marine bacteria associated with seaweed surfaces – K.G. Boyd & J. Grant Burgess
15.00	Antifouling steroids against larval settlement of barnacles from marine sponge and octocorals – Y. Tomono, H. Hirota & N. Fusetani
15.20	Incorporation of marine bacterial natural products into artificial surfaces and characterisation of their antifouling activity – E. Armstrong, K.G. Boyd, A. Pisacane & J. Grant Burgess
15.40	Evolutionary consequences of natural antifouling strategies; from bacteria to humans – J. Douglas McKenzie, M.M. Grundy, C.D. Bavington, N.V. Richardson, R. Lever & C. Page
16.00	Closing remarks

Abstracts



Oral Presentations

Biofouling and antifouling research in tropical systems: Indian Ocean studies and comparisons.

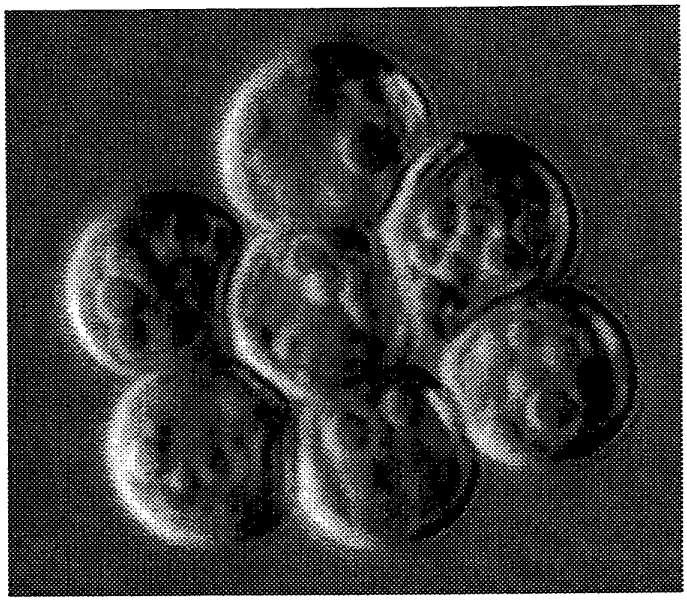
Arun B. Wagh[1] and Bernard J. Zahuranec[2]

[1]*Pune, India*
[2]*Office of Naval Research, Arlington, Virginia 22217, USA*

Comparison of biofouling, both microfouling and macrofouling processes, shows that while the same general principals apply in tropical, subtropical and temperate systems, tropical biofouling is faster both in settlement and in growth rates. Complexity of the fouling community may be greater in the tropics (though this is not always clear) but as in other systems, is strongly influenced by pollution effects with heavily polluted environments having degraded benthic communities. In common with other areas that have been studied, tropical biofouling communities are strongly influenced and affected by introduced or exotic species.

Examples of antifouling and biofouling community research in the tropics, especially in the Indian Ocean, will be discussed.

Session 1



Surface Characterisation and Corrosion

Microscopies, spectroscopies and spectrometries applied to marine corrosion

Brenda Little, Robert Pope and Richard Ray[1]

[1] *Naval Research Laboratory, Stennis Space Center, MS 39529, USA*

Surface analytical data were used to evaluate copper corrosion in marine environments. Recent developments in environmental scanning electron microscopy (ESEM) and transmission electron microscopy (TEM) equipped with electron energy loss (EELS) or energy dispersive x-ray (EDS) spectrometers make it possible to determine the concentration and distribution of elements associated with corrosion products and to resolve spatial relationships between microorganisms and corrosion products without fixation and dehydration. X-ray absorption (XAS) can be used to determine the speciation of some heavy metals, including copper associated with corrosion products. A unique feature of XAS is the element specificity which occurs because of the separation in energy of the absorption edges of different elements. X-ray absorption near-edge structure (XANES) provides information on metal site symmetry, oxidation state and the nature of the surroundings. Confocal laser scanning microscopy (CLSM) permits one to create three dimensional images, surface contours and accurately measure dimensions.

ESEM, CLSM, and TEM were used to demonstrate that marine bacteria are co-located with corrosion products, however this co-localization can not be interpreted as causal. In the absence of galvanic corrosion, three mechanisms are most often cited as causes for copper corrosion in marine environments: erosion corrosion, sulphide derivitization and copper concentration cells. ESEM coupled with EDS was used to study sulphate-reducing bacteria (SRB) associated with corroding copper alloys in seawater. Microbiologically generated sulphides preferentially reacted with iron and nickel in the alloys, resulting in selective dealloying. SRB distributed throughout the corrosion layers were encrusted with sulphides. The tenacity of the copper sulphide layers varied with alloy composition, but all were easily removed with turbulence. XANES spectra were used to demonstrate that copper from the substrata could be oxidized and bound within the biofilm as either Cu^{+1} or Cu^{+2}, but that once Cu^{+1} was bound, no further oxidation took place. TEM coupled with EDS was used to demonstrate localization of bacteria within marine biofilms and associated corrosion products. The combination of TEM and EELS allows identification of and discrimination between base metal and corrosion products. Used in combination, surface analytical techniques can be used to differentiate between abiotic and biotic corrosion, and accurately identify corrosion mechanisms.

The role of bacterial exopolymers in marine fouling and deterioration of steel surfaces

Rolf Gubner[1], Vitaly Zinkevich[1], Likit Hanjangsit[1], Iwona Beech[1] and Recep Avci[2]

[1]*University of Portsmouth, School of Pharmacy and Biomedical Sciences, White Swan Road, Portsmouth PO1 2DT, UK*
[2]*Department of Physics, EPS 259, Montana State University Bozeman, MT 59717-0350, USA*

The importance of extracellular polymeric substances (EPS) in bacterial attachment, biofilm development, and deterioration of steel is a subject of continuing discussion. Bacteria are commonly accepted to be the first colonisers during the fouling process. However, the precise mechanisms governing bacterial attachment to metal surfaces in marine environments are not yet fully understood. Our investigation aimed to elucidate the role of EPS in the initial adhesion of marine *Pseudomonas* NCIMB 2021 to surfaces of AISI 304 and 316 stainless steel. The latter were treated with three different types of exopolymers (planktonic, capsular and biofilm) produced by *Pseudomonas* NCIMB 2021 grown in a continuous flow bioreactor over a period of 30 days. Chemical analysis demonstrated that these EPS varied in their carbohydrate and protein content and composition.

X-ray photoelectron spectroscopy (XPS) analysis was undertaken to determine the effect of biofilms and the different types of EPS on the composition of passive layer of 316 stainless steel. Before exposure to bacteria, as well as prior to and following the biofilm removal, steel surfaces were imaged using techniques of scanning electron (SEM) and atomic force microscopies (AFM). Microscopy studies confirmed that damage to steel in the form of micropitting, occurred underneath the biofilm. XPS depth profile analysis revealed that the thickness and composition of passive layer (i.e., concentration of elements such as Iron, Chromium, Nickel, Molybdenum, Carbon, Nitrogen and Oxygen in these layers), varied between surfaces exposed to *Pseudomonas* and control coupons placed in sterile media.

Pre-treatment of AISI 304 and 316 stainless steel with the different types of EPS led to statistically significantly different attachment behaviour of *Pseudomonas* NCIMB 2021. Neither the difference in wetability of the steel surfaces nor the variation in surface roughness due to the EPS deposition could explain this phenomenon. It is therefore proposed that the chemistry of exopolymers influenced the initial colonisation of EPS-conditioned steel by *Pseudomonas* cells.

The use of infrared spectroscopy as a probe for monitoring the metal/biofilm/solution interfaces.

John Halsall, Maher Kalaji and Andrew Neal*

Department of Chemistry, University of Wales Bangor, Bangor LL57 2UW, U.K.

Infrared reflectance absorbance spectroscopy of exopolymers from bacterial exopolymers demonstrated differences in structure between capsular, free and biofilm exopolymers[1]. The molecular organisation of capsular exopolymers on hydrophilic and hydrophobic surfaces was monitored using the same technique[2].

The results indicated a preference for adsorption onto hydrophobic surfaces. Analysis of the data using 2D FTIR correlation showed that the adsorption mechanism and sequence of events are surface dependent[3]. The adsorption of polymers on both surfaces was shown to be a two-phase process, with the driving force behind the initial phase being driven by hydrophobic interactions.

1) I. Beech, L. Hanjagsit, M. Kalaji, A. Neal and V. Zinkevich; Chemical and structural characterisation of exopolymers produced by *Pseudomonas* sp. NCIMB 2021 in continuous culture; Microbiology, 1999, **145**, 1491-1497.
2) M. Kalaji and A. Neal; An infrared study of the self-assembly of capsular exopolymers from *Pseudomonas* sp. NCIMB on hydrophilic and hydrophobic surfaces; Biospectroscopy; submitted.
3) J.F. Halsall, A. Neal and M. Kalaji; Adsorption of bacterial capsular exopolymers to hydrophilic and hydrophobic surfaces; a 2D FTIR analysis; Biofouling; submitted.

* present address: Center for biofilm engineering, Montana State University-Bozeman, Bozeman, MT 59717-3980, USA

Characterization of a model conditioning film

P A Suci, G G Geesey, A M Baty

Center for Biofilm Engineering, Montana State University, MT 59717, USA.

The marine mussel, *Mytilus edulis*, produces a glue composed of various protein components that interact to form an adhesive plaque. One of the components, *Mytilus edulis* foot protein 1 (mefp-1) has been used as a model conditioning film. The following questions have been posed: 1) what are the molecular interactions involved in binding of bacterial extracellular polysaccharides to substrata conditioned with mefp-1; 2) can these interactions be influenced by the underlying substratum. The picture presented is incomplete, and at the same time contains ancillary information.

Mefp-1 has a number of properties that make it a suitable model compound. The protein, purified to homogeniety, is available. It is particularly well-characterized. It has been shown to adsorb strongly to a variety of surfaces and has been sold commercially as a coating to promote cell adhesion. Mefp-1 consists of a large portion of tandemly repeated peptide sequences. This increases the probability that structure/function relationships will be revealed by a given measurement technique.

Atomic force microscopy (AFM) of adsorbed films of mefp-1 shows that the film structure is different depending on the substratum to which the protein is adsorbed. The difference is very pronounced for adsorption onto polystyrene (PS) and poly (octadecyl methacrylate) (POMA). While the adsorbed film on PS consists of ovoid repeating structures about the size of one protein molecule, the film on POMA consists of structures that resemble irregular ridges of a mountainous terrain. Variable angle X-ray photoelectron spectroscopy (XPS) was used to determine the distribution of carbon (C), nitrogen (N) and oxygen (O) in each film. While the film on POMA is enriched in N at the film/substratum interface, the film on PS is enriched for N at the film/air interface. The data were interpreted as implying that cross-linking of the mefp-1 film occurs on the POMA surface, but not on the PS surface.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to characterize the interaction of the acidic polysaccharide, alginate, with adsorbed films of mefp-1. Binding of alginate to adsorbed mefp-1 was dominated by interaction with lysine residues. The interaction involves pyranose ring atoms of the alginate indicating possible coordination of the lysine to a number of atoms of the alginate. The interaction was not influenced by the underlying substratum to which mefp-1 was adsorbed.

The prosthecate marine bacterium, *Hyphomonas* (MHS-3) produces an extracellular capsular polysaccharide material that is a putative adhesin. Using ATR-FTIR affinity of an isolated fraction (fr2) of this material for a germanium (Ge) (oxide) film was quantified. It was shown that fr2 binds to the oxide through divalent cations. The affinity of fr2 for Ge was relatively high, but was decreased substantially if the Ge, PS and POMA were conditioned with mefp-1. This decrease in affinity of fr2 for conditioned substrata paralleled the adhesion behavior of whole cells.

In summary, masking of substrata properties by an irreversibly adsorbed protein conditioning film is a likely event if sufficient amounts of the protein are in solution. Some bacteria have evolved specialized extracellular polysaccharides to serve as adhesins. It is difficult to predict *a priori* if conditioning with a protein film will increase or decrease adsorption of bacterial polysaccharides.

Structural and chemical characterisation of echinoderm non-fouling surfaces.

Michelle M. Grundy[1], Dimitris Giantzoudis[1], Charlie D. Bavington[1], Neville V. Richardson[2] and J. Douglas McKenzie[1]

[1]*The Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, PO Box 3, Oban PA34 4AD, UK*
[2]*School of Chemistry, University of St. Andrews, Purdie Building, St. Andrews, Fife KY16 9ST, UK*

Many marine invertebrates have developed effective mechanisms for preventing biofouling. For example, the surfaces of echinoderms remain remarkably free from microfouling organisms, despite having an enormous surface area. The nature of the surfaces and the mechanisms by which adhesion of fouling organisms is prevented, is poorly understood.

By means of immunohistochemistry, electron microscopy (EM), atomic force microscopy (AFM) and Fourier transform infrared spectroscopy (FTIR), the physico-chemical properties of the surface cuticles of a variety of echinoderm surfaces have been investigated.

The cuticle of starfish and sea urchin tube feet and sea cucumber body wall were found to stain positively for anti-collagen type I and anti-chondroitin sulphate. FTIR spectroscopy experiments confirmed that the echinoderm cuticle was predominantly proteoglycan in nature and that its composition could be manipulated with enzymatic treatments, in order to elucidate which chemical components contributed towards its anti-adhesive properties. These studies have provided valuable high resolution, information about the structural arrangement of the echinoderm cuticle.

Studies of the cross-linking mechanisms of mussel adhesive proteins and barnacle cement

Kristin Mjörn[1], Camilla Fant[1], Fredrik Höök,[1,2] and Hans Elwing[1]

[1]*Department of Cell and Molecular Biology, Interface Biophysics. Göteborg University, Göteborg, Sweden*
[2]*Department of Applied Physics, Chalmers University of Technology, Göteborg, Sweden*

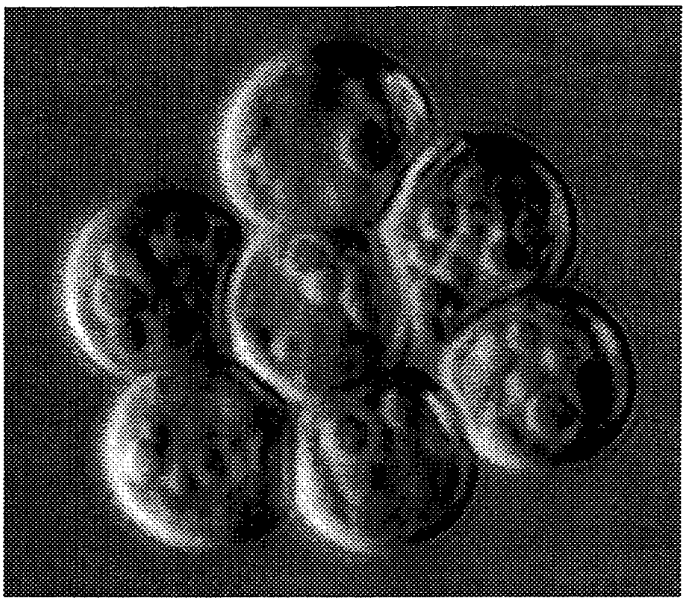
Introduction The attachment of marine organisms to solid surfaces is a research area of general importance in marine macrofouling. Of special interest is to understand and interfere with the molecular mechanisms of this attachment in order to develop surfaces with enhanced anti fouling properties.
There must be at least two conditions fulfilled for strong adhesion of the organisms to a flat solid surface. Firstly there must be sufficient strength of molecular adhesion at the liquid/solid interface. Secondly it is required that the adhered molecules are cross-linked at the surface and into the tissue of the organism. We have concentrated our effort to understand more about the cross-linking mechanisms. Unfortunately there are few methods available for measuring cross-linking of biopolymers in real time, which have made research difficult. Consequently, as a first step we have developed a methodological combination for simplified analysis of cross-linking of marine adhesives. Those methods will be presented as well as some results on the mussel adhesive protein (Mefp-1) from the common blue mussel, *Mytilus edulis*, and barnacle cement from *Balanus improvisus*.

Methods The marine adhesive proteins are adsorbed as monolayers on flat solid surfaces. Two different surfaces are used. One hydrophobic surface, consisting of gold functionalised with self-assembled methyl-terminated alkylthiols, and one hydrophilic silicon dioxide surface. Different cross-linking inducers are then applied on the monolayers. The process of adhesion and cross-linking is followed by two methods: Surface plasmon resonance (SPR) and Quarts crystal microbalance (QCM-D). With SPR, which is an optical surface sensitive method, we can determine the adsorbed amount of protein at the surface due to the difference in refractive index between water and the protein. With QCM- D, an acoustic method, we can determine the degree of bound water in the protein layer and the structural flexibility of the proteins.

Results In a typical experiment, mefp-1 was adsorbed to the surfaces and cross linking was induced by tyrosinase or by glutardialdehyde. Cross-linking was easily registered in real time as a loss of bound water and a decrease in flexibility in the protein monolayer. We can also conclude that the cross-linking effected the flexibility of the mefp-1 layer formed on the hydrophobic CH3-terminated surface much more than that formed on the hydrophilic SiO2-surface. We also made experiments with secondary cement from Balanus improvisus, but a single inducer of cross-linking has not yet been identified with the method used here. It is our hope that the described methodology will simplify and facilitate isolation of cross linking inhibiting compounds that can be used as antifouling agents.

Höök F, Brzezinski P and Kasemo B "Structural changes in Hemoglobulin during adsorption to solid surfaces: Effect of ionic strength and ligand binding" Proc Natl Acad Sci 1998, 95, 12271-12276
Fant C, Elwing H and Höök F "Adsorption behaviour and enzymatic induced cross-linking of a mussel adhesive protein" (submitted)

Session 2



Microfouling

The molecular and ecological diversity of bacterial adhesion to surfaces

Madilyn Fletcher

Baruch Institute, University of South Carolina, Columbia, South Carolina, USA

Despite over 20 years of intensive effort, researchers have been unable to identify a surface material that reliably resists bacterial adhesion in natural environments over long time periods of exposure. Although a number of materials may show greatly reduced attachment in the laboratory with test strains, they generally become colonized with extended exposure in natural environments. This failure of such potential anti-fouling surfaces is generally due to the remarkable diversity of natural bacterial populations, which exhibit a wide range of surface properties, attachment behaviors, and surface-colonizing strategies. Genetic and biochemical analyses have demonstrated that bacterial adhesives may comprise polysaccharides, proteins, and/or possibly lipids, depending upon the organisms. Moreover, different types of polymers may be involved at different times, when the attachment process occurs in stages. The numbers of attached bacteria and their adhesive strengths are also influenced by the chemical composition of the attachment substratum. Recent experiments utilizing chemically defined oligomers or self-assembled monolayers as substrata have demonstrated that the kinetics of bacterial interaction with the surface differs with the test organism and with the solid surface chemistry, but that these differences are no longer detectable after prolonged submersion in estuarine water. As bacteria colonize the surface, complex communities are developed, and the compositions of these assemblages can be influenced by the chemistry of the attachment surface. Recent studies utilizing genetic analysis of constituent organisms and analysis of data by neural network computing has allowed us to "fingerprint" total communities on specific surfaces and establish differences in mature biofilm communities. The complexity of these communities presumably arises from behavioral and physiological interactions, as well as the inherent attachment properties of the constituent organisms. Thus, the colonization of surfaces in natural environments involves an extremely complex and diverse array of molecular constituents and "recognition" interactions and responses, which combine to defeat any single approach to biofouling control.

The control of biofilms by signaling molecules and signal analogues

J. William Costerton

Center for Biofilm Engineering, Montana State University, Bozeman MT, USA

Bacteria in natural ecosystems have a very strong tendency to adhere to surfaces. Following their adhesion to a surface, these organisms undergo a transformation to a specific biofilm phenotype, and begin to form a biofilm by cellular replication and matrix formation. A mature biofilm, in a marine ecosystem, typically consists of +/- 85% matrix and +/- 15% cells. Direct examination of living biofilms, by confocal laser microscopy, has shown that biofilms are multicellular communities with a very elaborate architecture that indicates that their development must be controlled by signals analogous to the hormones and pheromones that control the development and behavior of higher life forms. We have discovered the identity of the signals that control the development of biofilms and cellular detachment from biofilms, in *Pseudomonas aeruginosa*, and these molecules are acyl homoserine lactones (AHLs). Mutants that cannot make these specific signals cannot form biofims, or detach from biofilms, respectively. Addition of the specific signal restores the activity in question. Because these signals interact with specific receptor proteins, their activity can be blocked by chemical analogues that react with the same proteins. A number of synthetic analogues have been identified that block biofilm formation, or induce biofilm detachment, and some natural analogues have been identified that have these same activities. Some of these natural analogues are produced by marine plants, that appear to use them to block the formation of microbial biofilms that would otherwise foul and occlude their surfaces. There is the very real and immediate possibility that signal compounds and signal analogues will be used to control the formation of deleterious biofilms in marine ecosystems.

Interactions between microbial biofilms and attachment of *Enteromorpha* spores

Ian Joint[1], Maureen E Callow[2] and James A Callow[2]

[1]*CCMS Plymouth Marine Laboratory, Prospect Place, Plymouth, PL1 3DH*
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This paper focuses on the initial stages of biofouling – the development of a microbial biofilm – and the hypothesis is tested that the presence of a microbial biofilm influences the probability of attachment by *Enteromorpha* spores.
Experiments have demonstrated that attachment of *Enteromorpha* spores is influenced by the presence of a microbial biofilm. Although *Enteromorpha* spores attach to glass when no microbial biofilm is present, the number of attached spores is significantly greater when bacteria are growing on the surface. A linear relationship was found between the number of attached spores and the number of bacteria per unit area. These results suggested that *Enteromorpha* spores might detect a chemical signal produced by the bacteria which influences attachment.

Image analysis has been used to quantify the spatial distribution of bacteria and attached spores. The experiments involved counting the numbers of bacteria and spores visible on glass slides. Since spores are much bigger than bacteria, when they attach, some bacteria are masked and are not visible. Analysis of the distribution of bacteria in the absence and presence of *Enteromorpha* spores is a way to investigate if spore attachment is linked to bacterial distribution.

Two alternative hypotheses were tested. Firstly, that spores attach to a surface as a result of a chemical cue produced by the bacteria. In this case, it is assumed that fewer bacteria should be visible on the slides than would be predicted by a random masking by spores. The second hypothesis is that the spores responded to variations in the micro-topography of the surface. That is, attached bacteria might change the micro-scale roughness of the surface and that there might then be more spores attached to areas with higher concentrations of bacteria. This hypothesis was tested by attaching inert microspheres to glass surfaces and comparing the distribution of *Enteromorpha* spores in the presence of bacteria or attached microspheres.

Bioadhesion and bioadhesives in marine raphid diatoms

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Diatoms (class Bacillariophyceae) are a diverse group of unicellular and colonial microalgae found in a wide range of habitats. Many forms of pennate diatom attach to a substratum and possess an active form of cell motility termed gliding. The ability of diatoms to adhere to a substratum and move is associated with an elongate slit, or raphe, that runs the length of each valve. The raphe provides an opening through the silica wall, permitting the passage of adhesive, mucilaginous strands that interconnect the cell and substratum and are somehow involved in generating cell movement. The secreted mucilage is left behind as the cell moves on, initially forming a diatom trail and eventualy a biofilm as the mucilage accumulates. Cells of *Craspedostauros australis* (formerly *Stauroneis decipiens*) typically settle on their sides (girdles), not on their raphes, and are only weakly attached in this position. Within one minute, most cells pull themselves up onto their raphes where they become tightly adhered to the substratum (i.e., first contact adhesion) and often become motile. These observations were used to develop a two-fold adhesion assay based firstly on the ability of cells to maneuver onto their raphes, and secondly to glide at the rate of control populations of cells.

We have detected a suite of extracellular proteoglycans that contain common antigenic epitopes and are located at the site of initial attachment of the diatoms to their substrate and in the trails of sticky mucilage that they secrete. The ability of StF.H4 antibody fragments to inhibit adhesion and motility suggests that the epitope bound by this antibody is likely to be close to the adhesive domain(s) of the molecule, and that this antibody should be useful in defining the adhesive site. The purification of the proteoglycans has been acheived by two-dimensional gel electrophoresis. All four molecules are protease sensitive, although preliminary results of amino acid analyses suggest that protein is a minor component. Current work is directed towards obtaining amino acid sequence from the four proteoglycans.

Structural and physical properties of hydrated diatom adhesives are being investigated with the Atomic Force Microscope (AFM). The AFM is being operated in contact or tapping mode under physiological conditions (in culture medium) to image the mucilage secretions, trails and biofilms produced by *C. australis*. The AFM is also being used to quantify the adhesive and elastic properties of adhesives by taking force curve measurements, and measuring the electrostatic charge of the mucilage secretions by assessing attraction/repulsion reactions with a silica sphere attached to the cantilever tip. The time taken for secretions to cure is being followed, as is the strength of adhesion.

We have taken the first steps toward characterizing a diatom adhesive, which is critical to our aim of understanding the mechanism of adhesion and how the processes of adhesion might be overcome. Once the determinants of adhesion are defined, we can compare them to a diverse number of organisms. If generic adhesion motifs exist within the range of bioadhesives, they may be used to develop new adhesives, or strategies to modify and/or prevent adhesion.

Interplay of surface chemistry, colonization promoters, diatom-diatom and diatom bacterial interactions in marine biofilm formation

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The illuminated fouled surface almost always bears a population of heterotrophic and autotrophic microorganisms, yet the majority of the literature on experimental biofouling involves heterotrophic bacteria only. There is a much smaller amount on diatoms and hardly any on the interplay between these organisms. However the literature on interactions between algae and bacteria in the water column is recognized as a definite niche called the phycosphere. In biofilms, cells are much more closely associated than in the water column, so that it seems that cell-cell interactions should be more likely. For instance we have shown that the metabolism of heterotrophic bacteria in a mixed algal/bacterial species biofilm under oligotrophic conditions is light dependent, thus demonstrating that interactions are possible. Here we describe other cell-cell interactions, cell-surface interactions and a simple method for demonstrating bacterial-algal interactions in biofilms.

The marine fouling diatom *Amphora coffeaeformis* has been found to attach to the clean surfaces of polymers and metals to a varying extent, emplifying the minimally adhesive range of about 22 dynes cm^{-1} known for other organisms. However when these data were plotted versus the polar component rather than the total surface energy of the surface, no minimum was seen. In any experiments of this type it is always a problem to separate physicochemical and structural chemical effects of the surface. With the help of the Materia Technica laboratory of the University of Groningen, we have studied solely the effects of the wettability of a surface. To do this, wettability gradients on glass surfaces were prepared. The only chemical differences along the gradient were the closeness of packing of methyl groups. Diatoms were found to attach along the gradient but only were motile where the contact angle was >40°. When measurements were carried out after a longer incubation time, the cells behaved as though the position of the gradient had changed so that the transition from hydrophobic to hydrophilic had moved in the direction of hydrophilicity, i.e., the cells may have conditioned the surface so that it was more amenable to their motile behavior and thus their ability to colonize. This possibility has particular importance in the design of low surface energy antifouling coatings.
Previously we have shown that diatoms can receive chemical signals and respond to them by changing their direction of motility. We have expanded this work using automatic dynamic image analysis to show that they also respond to auto-elaborated signals. Diatoms in colonies move away from the colony in a directed fashion. Their speed, direction and type of motility is controlled by the proximity of the colony. This could be exploited in the design of surfaces where adhesion was allowed, but subsequent further colonization was inhibited.

It is difficult to study the interplay of bacteria and diatoms on surfaces in a manner that is relevant to the production and maintenance of biofilms. We have designed a system that allows us to measure a parameter related to elaboration of the extracellular polymers that are the route cause of many of the problems associated with biofilms. The hydraulic conductivity of a glass bead-filled column is related to the degree to which the interstitial spaces become filled with organisms and their products, notably extracellular polymers. By measuring the hydraulic conductivity of such bead-filled columns, we have been able to assess which diatoms cause the most rapid reduction of flow and the effects of cell mixtures. The method, which allows many replicates to be processed simultaneously, is simple and inexpensive to carry out.

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Predation by protozoa on bacteria deposited on surfaces

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Predation by protozoa on suspended bacteria has been studied quantitatively many times, but studies on protozoan grazing of bacteria from surfaces are rare. Many protozoa, especially some flagellates and amoebae, have been seen to ingest bacteria from surfaces, often using special mechanisms, but such observations, and even counts of labelled bacteria ingested from surfaces by individual protozoa, give limited information on the effects of predators on bacterial populations, about the growth efficiency (GGE) of grazers and the likely extent of nutrient recycling that results from the grazing process.

The lack of quantitative studies on the grazing of bacterial populations from surfaces can be attributed to the absence of techniques for making quantitatively reproducible bacterial biofilms for use in such studies. We used three methods of depositing or attaching known quantities of bacteria upon surfaces, so that we could expose them to grazing by protozoa, and use methods previously developed to study grazing on suspended bacteria to measure features of grazing by different protozoa on bacteria from surfaces.

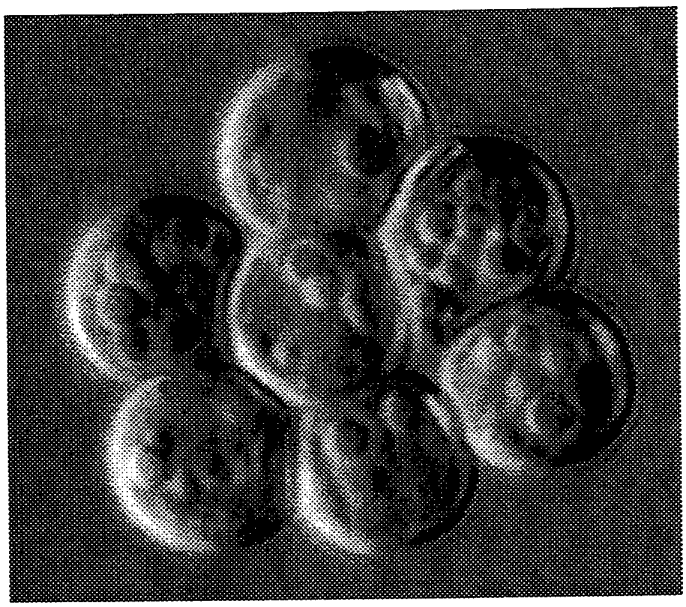
In method 1, stationary phase cultured *Vibrio* bacteria were deposited at the bottom of culture tubes by centrifuging. Small numbers of the surface-feeding flagellate *Caecitellus* were added, and the numbers of bacteria and flagellates, and the total protein in the tubes, were followed for up to 2 weeks, and compared with changes in tubes without flagellates. Similar experiments used *Bodo saliens*, which also picks bacteria up from surfaces, and *Cafeteria*, which collects bacteria from very close to surfaces. Grazing rates of these flagellates on deposited bacteria were compared with those on the same numbers of uncentrifuged bacteria by suspension-feeding protozoa: the flagellate *Pteridomonas* and the ciliate *Uronema*. Although the suspension-feeding protozoa grew more quickly than the surface-feeding ones, and consumed a higher proportion of the prey, the GGEs of all five protozoa were in the same range, and the number of prey consumed to produce one new predator varied in proportion to predator biomass. Surface-feeding protozoa are efficient bacterivores that graze a large proportion of the bacteria deposited on a surface.

In method 2, *Vibrio* labelled with ^{3}H thymidine and ^{14}C leucine then killed were stuck to albumen-coated PTFE membrane filters (0.4 μm pores) before exposure to grazing by *Caecitellus*, or the small amoeba *Vanella* (surface feeders), or by *Pteridomonas*. Grazing and assimilation of biomass by the protozoa were measured by changes in isotope distribution. Between the 5th and 15th days of incubation, *Vanella* consumed about 60% of the available bacteria with a GGE of 22%, compared with 75% consumption at 30% GGE for *Caecitellus* and 55% consumption at 17% GGE for *Pteridomonas*. The grazing and metabolism of the protozoa regenerated 70-83% of the nutrients present in their food.

In method 3, dual radiolabelled *Vibrio* or newly collected estuarine bacteria were filtered live onto aluminium oxide or polycarbonate filters and incubated to allow adhesion before exposure to grazing protozoa. The flagellates *Pteridomonas*, *Paraphysomonas* and *Cafeteria* and the ciliate *Uronema* all grazed suspended bacteria faster than deposited ones, but all grazed deposited ones to some extent. Flagellates grazed 2 to 5 times as many bacteria from the plastic filters as from the mineral ones.

We conclude that protozoan grazing can influence the build-up of bacterial populations on surfaces, both by removal of bacteria and by releasing nutrients that bacteria can use.

Session 3



Macrofouling

Substratum/bacterial interactions and larval attachment

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The role that microbial films play in the process of macrofouling is often confusing. Because they are present on almost every surface a settling larvae comes in contact with the microbial films can be a source of associative cues that may provide some information to the invertebrate. Although the film may consist of a variety of microorganisms, the focus of this presentation will involve the bacterial component. It has been demonstrated that the presence of a solid substratum presents an attaching bacterium with an altered microenvironment which can stimulate a variety of responses. In addition, a recent report indicates that quorum sensing molecules can have an influence on bacterial film architecture. These reports suggest that associative cues for larval settlement that are produced by bacteria may be generated in response to a substratum associated existence. Questions that arise are: Do specific surfaces stimulate specific bacterial responses? and, Do these responses influence larval attachment? Experiments involving films of the marine bacterium *Halomonas marina* and cypris larvae of the barnacle *Balanus amphitrite* suggest that the answer to both of these questions is yes. These experiments will be discussed along with a general overview of how the substratum may influence the bacteria that attach to it.

A field analysis of settlement of *Semibalanus balanoides* cyprids in response to substratum biofilming: the effects of diatoms, bacteria and grazers.

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Semibalanus balanoides displays markedly seasonal settlement around the British Isles, with peak settlement dates ranging between April and May according to location. Given that the spring period is one of intense of diatom growth, we investigated whether or not biofilms, and specifically the diatom component, might influence the settlement of *S. balanoides* cyprids in the intertidal. Our expectation was that photosynthetic components of biofilms might provide settling cyprids with cues to allow their discrimination of intertidal and sublittoral substrata. Settlement tiles were held under illumination in 6 closed recirculating tank systems and conditioned with biofilms in a 10°C constant temperature room for up to two weeks. Intertidal rocks were included in the reservoirs to 'seed' the tanks and the tiles were removed daily and held in air for 5 hours to mimic tidal emersion. Humidity was 100%. The composition of the biofilms was manipulated in two ways. First, germanium was used to inhibit diatom growth (3 tanks) and second, tiles in all 6 tanks were placed beneath either clear or black perspex boxes to provide lit and dark conditions. Conditioned tiles were allocated randomly to 3 horizontal frames (experimental 'blocks') placed in the barnacle zone and exposed to larval settlement for one tide (approximately 6 hours). Settlement was dense, at means of up to approximately 6 cyprids per square centimetre, and was apparently random within tiles. In contrast to expectation, the unmanipulated biofilms resulted in inhibition of settlement, in comparison to unfilmed control tiles, but the germanium-manipulated biofilms did result in settlement densities similar to those on the unfilmed controls.

The possibility that the apparent inhibitory effect of diatoms may actually be attributable not to diatoms, but to alterations to the bacterial component of the biofilms, was investigated further in a subsequent experiment by manipulating developing biofilms both in the light and the dark with antibiotics. The difficulties of generating ecologically realistic biofilms under controlled conditions in the laboratory and in the field are discussed, as are the effects of grazers on biofilm structure. The latter undoubtedly exert major influences on biofilm structure, although the extent to which those influences affect intertidal barnacle settlement remains unclear.

Barnacle settlement: field experiments on the influence of larval supply, tidal level, biofilm quality and age of *Balanus amphitrite* (Darwin) cyprids

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A set of three field experiments lasting 24 h was conducted during April 1998 at Duke Marine Laboratory (Beaufort North Carolina, USA) to: (1) assess the relative role of larval supply, intertidal height, quantity and quality of biofilm and age of the larvae on the settlement of *Balanus amphitrite* and (2) examine the correspondence between small-scale planktonic distribution of larvae, the initial spatial pattern of newly settled larvae and the vertical distribution of adult barnacles.

Precolonized methacrolate (Plexiglass) disks, arranged within three blocks, and established so as to eliminate edge effects within three large experimental panels placed at three predetermined tidal heights (High, Medium, Low) corresponding of to the upper limit, modal zone and the lower limit of adults of *B. amphitrite*. Split-Split-Plot ANOVAs were performed on densities of newly attached larvae (metamorphosis not completed) to test their habitat selection behavior to surfaces which had been precolonized by periphyton at 3 heights (origin factor) for 0, 7, 14 or 21 days (age factor). Physical environment (S, T, current flow) was stable and comparable during the three experiments. *B. amphitrite* cyprids were uniformly distributed in the water column. Larval supply was poorly correlated with the intensity of settlement over the one-week experimental period. In fact, the same larval supply could induce either a high (4X) or a low (1X) settlement after two tidal cycles and inversely, similar settlement intensity were associated with planktonic larval abundance varying significantly at a 2-day interval (109 to 171 cyprids. 923 l^{-1}).

Settlement was homogeneous on each experimental unit (no significant block effect). Tidal height, however, was a significant factor in determining the vertical patterns of newly settled larvae during the first experiment where larvae were abundant but not during subsequent experiments for which fewer larvae were collected.

The degree of periphytic precolonization was the main parameter affecting the settlement of *B. amphitrite*. For the two first experiments, 'weighed cyprid settlement' significantly decreased as the age of the biofilm increased revealing a strong preference of settlers for clean surfaces and avoidance of biofouled of all intertidal origins. Further analysis of biofilm samples showed that free-space availability and bacterial densities were significantly inversely correlated to settlement intensity. Moreover, settlement to 'favorable' substrata decreased by nearly ½ during our experimental period suggesting changes in the selectivity of settling larvae.

Our experiments confirm the role of larval supply in determining the vertical intertidal distribution of adults of *B. amphitrite*, but the short-term variability in the larval supply - settlement coupling observed over a one week period must be integrated in models of recruitment dynamics of barnacles.

KEY WORDS: *Balanus amphitrite*; barnacle; larval settlement; field experiments; larval supply, microbial biofilm; free-space availability; energetic contents.

Larval settlement and metamorphosis in the serpulid polychaete *Hydroides elegans* (Haswell) in response to cues from bacterial films

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Settlement and metamorphosis in larvae of *Hydroides elegans* are induced by certain bacteria in biofilms. Like in many other marine invertebrates, the nature of settlement cues that larvae of *H. elegans* acquire from bacteria remains unknown. In this study, we investigated basic properties of the settlement cues that larvae may acquire from bacteria in biofilms. Two strains of biofilm bacteria, which consistently promote settlement and metamorphosis of *H. elegans*, were used as model strains. Phenotypic data and sequences of 16s rDNA of the strains suggest that they belong to genus *Roseobacter* and alpha sub-group Proteobacteria.

Our results showed that larvae did not settle in waterborne bacterial metabolites and that high concentrations of bacterial metabolites were toxic to larvae. Bacterial films treated with formaldehyde or heat did not induce larvae to settle and metamorphose. Since both treatments were lethal to the bacteria and might impose damage to surface components of the bacterial films, we suggest that either viability of the bacteria or integrity of bacterial cell surface components, or both, is crucial to successful larval settlement. Further treatment of streptomycin caused a decline in population of viable cells in the bacterial films and simultaneously reduced the percentage of larvae settled on the bacterial films in a concentration dependent manner. Since streptomycin can kill bacteria without damaging bacterial cell surface components, the decline in larval settlement may be due to reduction in viable bacterial population in the bacterial films. Our current data suggest that the presumptive cues are not released into seawater and, therefore, should be bound on the surface of the bacteria. Further, the cues may be present or functional only when the bacteria are viable.

The effect of air drying a biofilmed surface upon larval settlement preferences of the tubeworm *Pomatoceros lamarkii* (Polychaeta: Serpulidae)

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The serpulid polychaete *Pomatoceros lamarkii* is a common organism on the rocky shores of north-western Europe. In the intertidal zone, adult and juvenile tubeworms are abundant around low water and occur higher on the shore predominantly on shaded and damp surfaces. Competent larvae of *P. lamarkii* settle and metamorphose in response to a marine biofilm and preferentially select surfaces supporting older films. In addition, the effect of adult worm 'leachate' (intact worms in seawater) in tandem with biofilms clearly showed the influence of existing adult worms on surfaces to larval settlement. These laboratory experiments are being repeated using natural intertidally produced biofilms to study the natural intertidal distribution of this common tubeworm.

Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitory cues for larval settlement in the polychaete *Hydroides elegans*

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In their natural environment many marine organisms are found to be free of fouling. This phenomenon indicates that these organisms may be able to produce compounds that either directly deter larval settlement of fouling organisms or allow the development of an unfavourable bacterial film on their surface to inhibit larval settlement indirectly. Therefore, the isolation of naturally occurring compounds with antifouling properties from these organisms has been considered as an alternative approach to toxic, non-targeting heavy metal-based antifouling paints.

The calcareous tube building polychaete *Hydroides elegans* is a major fouler in Hong Kong waters and is abundant in tropical and subtropical coastal fouling communities. In the natural environment, larvae of *Hydroides elegans* are induced to settle and metamorphose by contact to natural biofilmed surfaces. However, the non-damaged blades of the marine chlorophyta *Ulva reticulata* are completely free of fouling by sessile invertebrates. In laboratory settlement assays with *Hydroides elegans* larvae, we tested the hypothesis that this alga deters fouling through chemical means. Our results show a significant inhibitory effect of *Ulva reticulata*-conditioned seawater on larval settlement. Through ultrafiltration and chromatographic fractionation the algal exudates with biological activity were characterised as components with high molecular weight (above 100 kilo-Dalton).

We will present the results of a bioassay-guided purification procedure, laboratory larval settlement assays with different size fractions of the algal exudates as well as their impact on single bacterial strains that induce larval settlement in *Hydroides elegans*.

Understanding settlement and primary adhesion in *Enteromorpha*

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Novel solutions to non-toxic control of fouling organisms depend on a greater understanding of attachment processes. Fouling of surfaces by the green alga *Enteromorpha*, involves a transition from a motile spore to an adhered, sessile spore. Attachment is considered to involve *settlement* processes including cue detection, surface selection and temporary adhesion, all of which may be moderated by the microbial biofilm. The spores then irreversibly commit themselves to *primary adhesion* during which a glycoprotein adhesive is secreted from vesicles contained in the anterior region of the swimming spore. This paper describes aspects of our current programme exploring the mechanisms of settlement and primary adhesion. To characterise molecules involved in adhesion, monoclonal antibodies have been raised against settled spores displaying the adhesive. Candidate antibodies have then been selected by ELISA, Western blotting, immunofluorescence and immunogold microscopy. The antibodies have been used as molecular probes to identify an antigen which runs as a 110 kDa N-linked glycoprotein in reducing-PAGE, but as a series of higher molecular weight components under native conditions. The involvement of this antigen in primary adhesion has been confirmed by functional inhibition assays. The putative adhesive molecule appears to be closely related to cell wall proteoglycans. Molecular characteristics of this antigen will be discussed.

Towards an understanding of gregariousness in barnacles

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It has been known for almost 50 years that gregarious settlement behaviour of barnacles has a chemical basis. Early workers in the field also recognised that a mechanistic understanding of this process would be of value to antifouling. The studies that followed on *Semibalanus balanoides* provided strong evidence of a proteinaceous inductive cue of adult, cuticular, origin and of a perception system that involved a tactile chemical sense and increased tenacity of adhesion by the settlement stage cypris larva.

Because it is a fouling species of major economic importance and a model for invertebrate larval settlement studies, we have, in common with other recent investigators, focused on *Balanus amphitrite* rather than *Semibalanus balanoides*. Like *S. balanoides* the inductive cue to settlement of *B. amphitrite* is a glycoprotein. The cue is composed of three major subunits of similar biological activity and for this reason it been termed has the 'settlement inducing protein complex' (SIPC). Using a modified protocol, SIPC and its component subunits have been isolated for immunological studies and further characterisation.

In contrast to reports for *S. balanoides*, the carbohydrate moieties of *B. amphitrite* SIPC contribute to its activity. The oligosaccharide side chains of each subunit are N-linked and differences in sugar composition have been detected. These findings have prompted a re-examination of earlier claims regarding the nature of the settlement pheromone, 'arthropodin', of *Semibalanus balanoides*.

Settlement induction by SIPC is not species specific but the magnitude of the effect is greatest for the conspecific. This finding suggests relatedness in molecular character and chemosensory recognition involving receptor-ligand interactions. Significantly, western blots, using a polyclonal antiserum to the 76 kDa subunit of *B. amphitrite* SIPC, have detected SIPC-like activity in all 13 species examined thus far. Slight differences were, however, detectable in molecular size and staining intensity of the reactive bands. Not surprisingly, therefore, a monoclonal antibody specific for the 76 kDa subunit of *B.amphitrite* SIPC failed to detect activity in *Elminius modestus* extracts.

Taken together, our findings suggest that SIPC-like proteins are ubiquitous to balanomorph barnacles. While differences in molecular character of the major subunits which comprise SIPC have been detected, such differences do not appear to contribute in a major way to gregariousness, but may be important to discrimination between species at settlement. An antifouling strategy that operates at the level of receptor-ligand interactions may thus be feasible for barnacles in general.

Prostanoids and their effects on larval settlement in the barnacle, *Balanus amphitrite*

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Eicosanoids are C20 fatty acid derivatives with a wide range of functions. There are two main types; the prostanoids such as prostaglandins (PG), and thromboxane (Tx) and the leukotrienes and lipoxins. Recent renewed interest in the nature and functions of eicosanoids in invertebrates has resulted in several key observations of general evolutionary interest.

We have examined both the nature and function in larval settlement of eicosanoids generated by both larval and adult tissues in the barnacle, *Balanus amphitrite* and the sea squirt *Ciona intestinalis*. Both organisms have a complex profile of eicosanoid generation. For example, in *C. intestinalis* we found the generation of several monohydroxy fatty acid derivatives including 8-hydroxyeicosapentaenoic acid (8-HEPE), 12-HEPE and 5-HEPE (Knight *et al.*, *Biochim. Biophys. Acta*, **1436**, 467-78,1999). These animals also synthesised several dihydroxy fatty acid derivatives such as 8,15-dihydroxy eicosapenatenoic acid (8,15-diHEPE). Full structural eludidation of these major compounds was achieved by mass spectrometry. In *B. amphitrite* the eicosanoid profile differed. Here, as well as the monohydroxy fatty acid derivatives, we observed a novel group of products containing a conjugated pentaene backbone. These are the subject of further biosynthetic and functional studies. Both organisms also produced prostanoids including $PGE_{2/3}$.

Further experiments were carried out to determine if these eicosanoids had any effect on larval settlement and metamorphosis. In these we mainly employed larval *B. amphitrite* due to their availability and reactivity *in vitro*. Synthetic PGE_2, PGE_3 and the stable prostaglandin analogue, 16,16-dimethyl PGE_2, all had a dose dependent inhibitory effect on larval settlement. Furthermore, the selective PG biosynthesis inhibitor, indomethacin, stimulated larval settlement in a dose-dependent manner.

These experiments give insight into the potential role of eicosanoids in larval settlement. Further studies to be discussed are aimed at examining the mechanism of their action in particular the interaction of PGs with other secondary messengers.

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Are differences in settlement generated by variation in the exploratory behaviour of cyprids?

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Numerous settlement studies have been carried out on a range macrofouling species, and within-study comparisons have led to the documentation of a wealth of factors that affect settlement. However, comparison between such studies is confounded due to the use of different settlement substrata and experimental methodologies, and thus the identification of generic factors mediating settlement is limited. To permit valid between-species comparisons of settlement, an investigation was carried out to determine differences in the settlement strategy of four common barnacles using comparable methodologies. Replicate precise and accurate settlement tiles of four textures (smooth, fine, medium and coarse) were deployed during the settlement season when relatively monospecific settlement could be obtained. The target species were *Semibalanus balanoides* (Clyde Sea, UK), *Balanus amphitrite* (Genoa, Italy), *Balanus eburneus* (Florida, USA) and *Balanus improvisus* (Rostock, Germany). The results showed that all four species displayed a settlement response mediated by substratum texture, but that the response differed between species. This suggests a range of settlement strategies, though the reasons for this difference remain unclear.

The key link between larval supply and larval settlement is the phase when the larvae is exploring the substratum. However, this phase in the life–cycle, moving from the water column to the hard substratum, has been little studied. Consequently, a study was carried out in the Clyde Sea to document the exploratory behaviour *of S. balanoides* cyprids in the field. A high-resolution, colour underwater video camera was used to record the exploratory behaviour of *Semibalanus balanoides* cyprids *in situ*. Larval supply, in terms of the number of cyprids encountering the surfaces varied in terms of numbers and pattern. A total of 1014 cyprid exploratory tracks were digitised and the time spent on the surface, distance travelled and track characteristics were recorded. Exploratory behaviour was filmed at night using a far-red light and compared to the daytime behaviour, and differences were found in exploration duration and distance. In addition, a difference in the angular orientation of exploratory behaviour was found between day and night. These results show that cyprids can display a wide range of exploratory behaviours. It is argued that only through an understanding of the differences in exploratory behaviour can the causes for differential settlement patterns be understood.

Surface texture within a narrow range eliminates settlement of the barnacle Balanus improvisus

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Experiments performed in the field and in the laboratory support the observation that surface texture has a negative influence on settlement of the barnacle *Balanus improvisus*. Settlement of *B.improvisus* was evaluated on designed surfaces with different scales of surface texture. Surface texture within a narrow interval (20-40 μm) reduced settlement by 95 % compared to smooth surfaces. Experiments in progress are designed to understand the behavioural mechanisms by which cyprids reject textured surfaces and ultimately which cues are used in making this desicion. In addition, hydrodynamic measurements conducted on panels with a specified surface texture (e.g. riblets) show that for a vessel speed of approximatly 20 knots, the surface friction is reduced within the same interval as the reduction of barnacle settlement.

Attraction and deterrence: settlement behaviour bioassays for the screening of non-toxic coatings

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Conventional toxic coatings are easy to test, they either kill settling organisms or they do not. Thus in the laboratory, standard LD_{50} or LC_{50} or similar tests can be used and field trials rapidly show whether or not the coating prevents settlement. For toxic coatings, negligible settlement constitutes failure, however non-toxic coatings rarely give such clear results and coating developers are normally seeking a marginal reduction in fouling. Scoring and analysing such field trials is demanding on facilities and time, and requires carefully controlled experiments coupled with sophisticated statistical analysis. Obviously laboratory lethality tests do not work for non-toxic coatings. Consequently, the development of non-toxic coatings is being hampered by constraints imposed by testing of their efficacy. The aim of this project was to develop techniques for the videoing of behaviour of cypris larvae of *Semibalanus balanoides* in the field. The objectives were to use the technique to film larval explorers on surfaces a) with or without settlement factor, b) treated with antifouling coatings, c) with or without larval footprints, and finally, d) to develop a behavioural bioassay for non-toxic antifouling coatings.

An underwater camera system was used. This consisted of a remotely controlled colour camera with a zoom lens, a surface control unit, and 30m of umbilical cable. A Hi8 VCR was used to monitor and record the images. All fieldwork took place at Millport, Isle of Cumbrae, Clyde Sea during the settlement of season of *Semibalanus balanoides*. Nitro-cellulose membranes with identified spots of purified pheromone from either *Balanus amphitrite* or *Semibalanus balanoides* were filmed. Out of 211 cyprids filmed only 1 settled (~0.5%). There was no difference in track length, crowflies length or straightness between the two treatments, but cyprids spent more time exploring, and at a higher speed, on *Semibalanus* treated membranes. Angular activity was significantly higher in tracks encountering pheromone areas, than those missing them. Thus it appears that although the two pheromones generate novel behaviour, there is some specificity exhibited by *Semibalanus balanoides* cyprids to their own pheromone. To determine if crude footprints (*viz* remnants of antennular secretions which include a pheromone component) left by previous cyprid explorers could change the behaviour of subsequent explorers, the length of time cyprids spent on a surface was analysed with respect to the time of arrival. The analysis of the effect of footprints was equivocal. Undoubtedly footprints must have some effect as the response to the purified pheromone is very clear, yet the response is easily masked by behavioural changes caused by other surface parameters. Three antifouling coatings were examined: a conventional marine tin-based self polishing copolymer, a non-toxic silicone-based coating, and a non-toxic coating of unknown chemistry. Both the tin and silicone coating demonstrated clear deterrence of larvae, but the other coating exhibited neither deterrence nor attraction. Subsequent immersion trials have shown that the latter coating fouls. This work on antifouling coatings has demonstrated that the analysis of larval behaviour is a subtler bioassay than settlement or immersion studies, and with suitable computerisation it is a very rapid means of screening non-toxic coatings.

An overview of the key achievements of the Fusetani Biofouling Project

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Fusetani Biofouling Project was one of the five-year basic research programs of Exploratory Research for Advanced Technology (ERATO), Research and Development Corporation of Japan (JRDC; present name: Japan Science and Technology Corporation or JST which is one of the statutory corporations under the Science and Technology Agency, Japan). Research of the Project was started from October 1991 and terminated at September 1996.

We focused on larval settlement and metamorphosis, extremely important components in understanding the interactions between marine organisms. The functions of chemical substances were investigated concerning both the receptors and second messengers as parts of these particular phenomena.

The major research themes were as follows:

1) Larval culture of sessile marine organisms (the mussel *Mytilus edulis galloprovincialis*, two barnacles *Balanus amphitrite* and *Megabalanus rosa*, the hydroid *Tubularia mesembryanthemum*, and the bryozoan Bugula neritina)
2) Imaging analysis of larval behaviors during settlement
3) Chemical cue to settlement in barnacles
4) Signal-transduction systems and cementing in barnacle larvae
5) Antifouling compounds against barnacle larvae
6) Exogenous/endogenous factors for settlement of actinula larvae
7) Characterization of bryozoan larvae
8) Metamorphosis-inducing substances of ascidian larvae
9) Microbial films

We have published almost 50 original papers, and 30 review articles and books, in addition to 20 patents. After termination of the research period on the Project, the research on chemical cue to settlement in barnacles has been developing vigorously in Clare's laboratory, MBA. Most of the other themes have been also studied in various styles.

Ship hull fouling as vector of species introductions

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Ships have been recognized as a major vector for the introduction of non-indigenous and harmful organisms. During the last decades ballast water discharges have increased throughout the world in most of the major ports. But ballast water is not the only vector of unintentional species transportation with ships.
During a joint research project between the Institute for Marine Sciences, Kiel and the University of Ham-burg commissioned by the German Environment Protection Agency (Berlin) the ballast water, tanks sediment and hull fouling of nearly 200 vessels have been sampled. Found species ranged from microalgae to 15 cm long fishes. Of the 404 species identified, approx. 60 % were classified as non-indigenous species to German waters. Non-indigenous species were recorded in 37 ballast water (37,8%), 30 sediment (56,6%) and 126 hull samples (97,5%).

An assessment was undertaken to identify the risk of unintentional future species introductions with ships. The potential for establishment was classified into 3 categories according to matching climatic and salinity conditions in the area of origin compared with those in the port of destination. Ballast water is estimated as an important vector for future introductions of non-indigenous species in our waters, but most of the species with the highest potential for establishment were recorded in hull samples and not in ballast water or sediment samples.

A recent (1998) summary of non-indigenous species in coastal waters of the North Sea revealed about 80 exotic species in self-sustaining populations. The majority of introduced species in the North Sea are invertebrates; predominantly crustaceans, polychaetes and molluscs. Ships are assumed to be the most important vector (approx. 45 introductions). Hull fouling seems to be a more important vector (27 species) compared to ballast water (18 species). Even a new species to science (a turbellarian) was found in hull samples of one ship.

It was concluded that each vessel from overseas is a potential carrier of non-indigenous organisms in sufficient numbers to establish a founder population in the North Sea. Since even a single introduced non-indigenous species may cause severe damage, it is necessary to develop preventive measures for unintentional species introductions. These treatment methods should not focus on ballast water mediated introductions alone, but should take into account introductions by hull fouling of ships as well.

The effects of marinas on the distribution of macro-fouling organisms around Melbourne, Australia: observation, experimentation and speculation.

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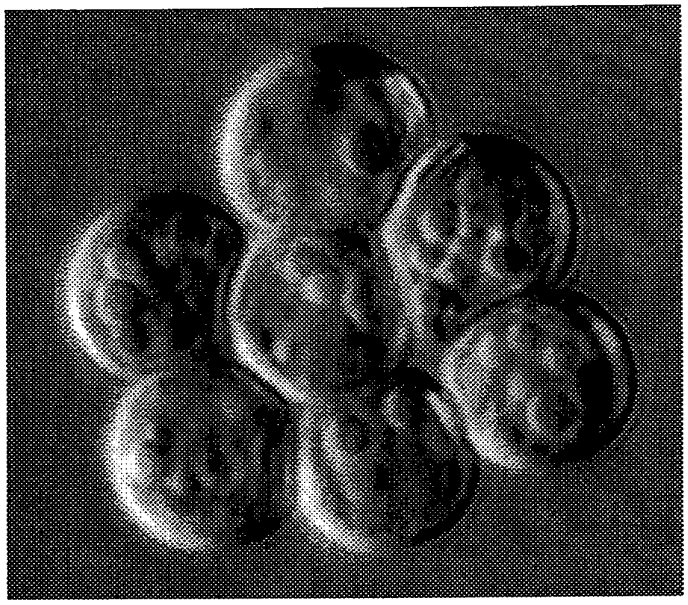
We examined faunal distributions around two marinas near Melbourne, Australia. Fouling panels were deployed for three months inside each marina, and at six nearby reference sites. Marinas had a major effect on assemblage composition. Plates deployed inside the marinas were dominated by solitary ascidians, whereas those from the reference sites were dominated by encrusting and arborescent bryozoans. We also examined the distributions of recruits on panels immersed for two weeks. Encrusting bryozoans were found in greater numbers at reference sites as compared to the marinas, mimicking the patterns seen in the three month old panels. Solitary ascidians and arborescent bryozoans did not show consistent patterns of recruitment. A reciprocal transplant of plates between marinas and reference sites showed decreased survivorship of arborescent bryozoans when they were moved into marinas. Solitary ascidians were unaffected by transplantation, and encrusting bryozoans suffered a general die-off at all sites.

We hypothesised that the lowered numbers of bryozoans found in marinas could be due to the presence of toxicants in these waters. Using copper as a reference toxicant, we performed field dosing to examine responses of the taxa. Copper decreased the abundance of encrusting bryozoans on fouling panels, but did not affect the settlement rates of this group. Paradoxically, the abundance of arborescent bryozoans was increased the presence of dosed copper at three out of four sites, but the settlement rate was unaffected. Dosed copper caused also increased settlement of solitary ascidians at two sites, but overall abundance on panels was unaffected.

Session 4



Towards Alternative Technologies to Metal-Based Antifouling Coatings

Towards non-toxic fouling-resistant coatings design and preparation of low-surface energy coatings with controlled surface chemistry and microstructure

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A controlled biological response is dependent on the physical and chemical surface properties of the material. It has been shown, for example, that surface microstructures can affect both cell growth and cell response. The synergetic effect of directed biological response and low bioadhesion should be thus utilized for solving marine fouling problems. Sol-gel chemistry based on polydimethyl siloxanes has been found to be very attractive for preparing non-toxic coatings that exhibit minimal adhesion by marine organisms. In this study, we have investigated the effect of micro texture on the biofouling resistance of low surface energy RTV silicones (PDMS). Surface microtexture in the range of 50 – 100 μm was achieved by molding silicones against various mesh structures. All surfaces were characterized with ESCA, SEM, AFM and dynamic contact angle measurements. Above a certain degree of surface roughness, the wetting behavior of surfaces has significantly changed. The water contact angle hysteresis decreased, which is characteric for composite surfaces. Field studies on the west coast of Sweden showed that the micro-textured PDMS surfaces were significantly less fouled by the *Balanus improvisus* barnacle than the smooth control PDMS surfaces.

Self-assembly and phase separation of polymers have recently been pointed out as attractive techniques for preparing structures of controlled size and morphology. Surface segregation of fluorine-ended monomers and fluorinated block copolymers has been employed for engineering the surface chemistry and microstructures. We have demonstrated that the surface chemistry and surface morphology of a sol-gel system composed of poly(dimethylsiloxane) (PDMS) and (tridecafluoro-1,1,2,2-tetrahydrooctyl) triethoxysilane (FTEOS) cross-linker can be controlled by varying the catalyst concentration, molecular weight of PDMS and humidity during curing. ESCA analysis revealed that increasing the catalyst concentration and humidity during curing resulted in up to eight times enrichment of fluorine at the surface. The variation in surface topography from smooth to micro-structured was visualized with AFM. The structures formed mainly with low molecular weight PDMS and at medium to high catalyst concentrations were found to be in the range important for directing the biological response.

Nontoxic fouling release: correlation of polymer surface properties with ease of fouling removal

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Nontoxic fouling release coatings are of interest from both environmental and economic standpoints. Such coatings exhibit minimally adhesive surfaces with respect to biofouling. This paper attempts to correlate physical surface characterization data on silicone "fouling release" polymer coatings with measurements of the tenacity of adhesion of hard fouling in the marine environment. Our hypothesis is that design criteria for the optimal fouling release coatings include: (1) a low surface energy to minimize chemical interactions; (2) a low glass transition temperature, Tg, to minimize mechanical locking of a prospective fouling organism; and (3) temporal and chemical stability in water. That is, conditions (1) and (2) must not change with immersion time in water. This paper addresses the success in meeting these criteria for two well known PDMS classes, namely *alkoxysilane* cured networks and *Pt cured* networks. The importance of temporal stability of surface properties is emphasized in the optimization of fouling release. Progress toward the development of a fouling release coating with barnacle adhesion (estimated by the method of Swain) between 5 and 6 psi will be described.

The performance of fouling-release coatings: static immersion at seven sites worldwide

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The Office of Naval Research (ONR) and the Office of Naval Technology (ONT) have supported a program to develop non-polluting antifouling. At present, the most promising of these are the silicone-based fouling-release coatings. It is recognized, however, that the performance of these systems can vary with geographic location. To investigate such effects, four non-toxic surfaces General Electric RTV11 (GE3), Dow Corning 3140RTV (RTW), International Intersleek (IN5), and F152 epoxy (15W) were exposed to biofouling at seven locations around the World. The experiments were designed to investigate:
variations in coating performance within individual test sites
variations in coating performance among different test sites
variations in coating performance among coating types

Three replicates of each coating were exposed at the seven static immersion sites located in California (CA), Florida (FL), Hawaii (HI), Hong Kong (HK), India (IN), Italy (IT), and Singapore (SG). The exposure commenced in April 1996 and continued until September 1997. The surfaces were monitored for physical condition, biofouling development (as defined by percent cover of biofouling attached to the coating), and biofouling adhesion (as defined by water jet pressure to remove slime films and shear force required to remove hard fouling). This paper details their performance and condition over the first sixteen months of exposure. The surface properties of the coatings were characterized using contact angle analyses, multiple-attenuated internal reflection infrared spectroscopy, nylon brush abrasion tests and cross sectioning at the Center for BioSurfaces, SUNY Buffalo

The data showed that the relative performance of the coatings was similar among test sites. However, the quantity and species composition of fouling organisms varied worldwide. Adhesion strengths of hard fouling also varied with respect to species and coating type.

It is recommended that future research be directed to increasing our knowledge of adhesion strengths and adhesion mechanisms of different organisms to fouling-release surfaces. This should include both larval and adult phases. Such information will help in the formulation of improved fouling-release coatings and in the prediction of their performance when applied to ship hulls.

Temporal and spatial variations of macro-fouling on silicone biofouling release coatings

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Macrofouling is a ubiquitous problem in the aquatic environment. The effect of biofouling on the hulls of vessels has been shown to reduce maximum speed and increase propulsive fuel consumption. Traditional antifouling paints containing toxic triorganotin compounds or cupric oxide are highly effective in controlling the fouling. Due to environmental concerns, the application of triorganotin based paints has been prohibited and it is expected that the use of cupric oxide paints will be limited in the near future. Oftentimes, the most insidious macrofouling organisms are barnacles, oysters and tubeworms or in fresh waters, zebra mussels.

Nontoxic, low surface free energy silicone coatings with reduced biofouling adhesion have been developed as an alternative to antifouling paints. Tests have shown that silicone coatings permit macrofouling to attach, however, the fouling can be removed either with water pressure or by scrubbing with a brush.

Community structure and fouling coverage are important elements for performance evaluation of non-toxic silicone release coatings. Long term fouling coverage data have been collected at three sites on oil amended silicone coated panels. The test sites are located offshore of Massachusetts, Hawaii and Florida. For each coating, intersite differences in fouling coverage and community makeup are observed. Intrasite temporal changes of the fouling for each panel will be described. The effect of coating composition on fouling coverage is discussed.

The surface properties of some silicone and fluorosilicone coating materials immersed in seawater

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The resistance to marine biofouling of a low-surface-energy material may be reduced by changes in the physical characteristics of the surface. Possible changes including adsorption of a conditioning layer, formation of a biofilm, absorption of water and surface conformational changes induced by interactions with seawater tend to reduce the surface energy so that the material may become susceptible to microfouling and macrofouling.

A bubble-contact-angle method has been used to monitor interfacial free-energy changes over periods of up to 6 weeks, for some commercial silicone elastomers and synthesised fluorosilicone elastomers in distilled water and seawater (artificial, filtered, unfiltered). This method enables the surface properties of the immersed materials to be monitored, under conditions appropriate for microbial colonisation. Bacterial settlement has been visualised by scanning electron microscopy. The uptake of water has been determined gravimetrically.

Immersion in water (all sources) progressively reduced the contact angles in all silicones by up to 70° over the full period. Changes were more rapid in natural seawater and were slower for fluorosilicones. The silicones absorbed water (up to 1.1%). Observations using SEM showed that a contact angle of 20–30° was consistent with the formation of a layer of attached bacteria and microalgae. The relationship between surface properties and the susceptibility to microfouling will be discussed.

The roles of surface energy and frictional processes in release phenomena

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We have investigated the adhesive and tribological properties of a number of commercial silicone coatings using contact mechanics, slip and peel tests. These studies demonstrate that the release properties of a polymer do not correlate with its surface energy in a simple way. The release properties of the coatings, on the other hand, correlate well with their tribological properties. We have now developed a study using model PDMS networks, the properties of which are modified with silicone oil. The tribological properties of these coatings are influenced significantly, when the molecule is larger than the mesh size of the elastomer. Further studies based on proteins as well as self-assembled monolayers have thrown new lights on the science of release mechanism that are of importance to the design of polymeric coatings that would facilitate mechanical release of organisms in biofouling environments.

Preventing the bacterial colonisation of surfaces: the non-stick-coating approach

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The strategies for attachment adopted by marine bacteria and other fouling organisms vary according to the nature of the surface and the microenvironment. Colonies of settled bacteria represent a food source for microalgae and macrofouling and also provide the supporting substrate for their attachment. An alternative to chemical attack on established colonies (bleaches, detergents) and to toxic surfaces (antibiotics, copper, organotin additives to paints) is to provide surfaces of environmentally-friendly, non-toxic polymeric materials onto which these colonisers will not adhere; essentially to inhibit settlement by removing the ability of the surface to form a permanent bond with the microorganism.

The attractive forces generated by the adhesive secreted by the bacteria must be compatible with those of the surface being addressed. For a polymeric material, these are the van der Waals forces and sometimes also electrostatic interactions; minimisation of these cohesive forces enhances the non-stickiness of the coating material.
We have recently prepared the first generation of such polymers. These materials are non-toxic and, films formed from them have been shown to be resistant to all microorganisms against which they have been tested. At the molecular level, the coatings are made up of chemical entities analogous to those found in acrylic paints and silicones but, by replacing some of the hydrogen atoms with fluorine, the electrostatic interactions become negligible and the van der Waals' attractions are minimised, making the materials very slippery.

Methods for the fabrication of smooth film structures will be described and their determined physical characteristics will be presented. Preliminary observations of their resistance to some marine fouling organisms, including bacteria, *Enteromorpha* and barnacles, will be considered on the basis of their surface energy characteristics.

Mechanism of barnacle removal from silicone coatings

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Silicone appears to have the best release property against hard biofoulants of all the "low surface energy" easy-release polymers. The NRL silicone duplex coating has the excellent release property of silicone as well as the toughness and bond strength required of a rugged ship hull coating. The duplex coating consists of an outer layer of a commercial silicone and a bond coat of 60% silicone and 40% butyl acrylate styrene. The effective elastic modulus ($E^*=E/(1-\nu^2)$, where E is the Young's modulus and ν is the Poisson's ratio) of the coatings, measured by continuous indentation, were 3 MPa and 23 MPa, respectively. Mechanisms of barnacle removal from silicone coatings can be studied in the laboratory by pull tests using epoxy-coated studs, referred to as pseudobarnacles (PBs). In this study, PBs were removed from duplex coatings using a commercial stud pull tester. Coatings were prepared with bond and top coat thicknesses ranging from 25 μm to 700 μm. The pull-off force required to remove a PB *decreased* as both top and bond coat thicknesses *increased.* This behavior is consistent with Kendall's model for removing stud from elastic glue layers [K. Kendall, J. Phys. D: Appl. Phys., **4**, (1971) 1186]; the model was extended to account for thickness and bulk modulus of both layers of the duplex coatings. The mechanism of release, investigated by video inspection of PBs removed from clear silicone coatings, was found to be peeling, as proposed by Kendall. Issues of surface energy, adhesion and removal processes will be reviewed and discussed. Finally, preliminary observations from pull-off studies of Chesapeake Bay barnacles from clear silicone coatings will be reported.

J.G. Kohl and I.L. Singer, "Pull-off behavior of epoxy bonded to silicone duplex coatings," Progress in Organic Coatings, 1999

Surface active neurotransmitter antagonists prevent the settlement of cyprid larvae

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The barnacle *Balanus improvisus* is the major fouling organism in Swedish waters and it colonizes most manmade surfaces submerged in the sea. Thus, it is of great importance, both environmentally and economically, to develop a non-toxic method for the inhibition of barnacle settlement.

Several neurotransmitter antagonists have shown to be very efficient in preventing settlement of cyprid larvae of both *Balanus improvisus* and *Balanus amphitrite*. Experiments have been carried out on lab reared cyprids in petri dishes of hydrophilised polystyrene, using primarily adrenergic antagonists dissolved in seawater. Two of these drugs repeatedly inhibited settlement at concentrations between 25 pg ml^{-1} and 2,5 ng ml^{-1}. These substances display a low toxicity, the lethal effect occuring at 25 µg ml^{-1}. Experiments have also been carried out to examine the reversible effect of these compounds. Cyprids incubated with substance XH11 (subject for patent pending) for 6 h did not settle after being washed and transferred to seawater. A striking feature of these substances is their strong tendency to accumulate in solid/liquid phase boundaries. This ability makes them particularly attractive candidates for the development of slow-release carriers in marine coatings.

Chemical ecology of seaweeds; surface-based interactions.

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In contrast to the chemical ecology of marine plant/herbivore or predator/prey interactions, studies of analogous surface-based interactions in the sea have proceeded with relatively few unifying ecological themes. In order to try and understand whether there are generalities in the way in which chemical signals are used to mediate surface-based interactions in marine systems, we have studied - for a few selected systems - both inhibition and facilitation of colonisation for both eukaryotes and prokaryotes. Our best studied example of these processes is for the Australian red alga *Delisea pulchra*, which produces a structurally related set of non-polar metabolites known as halogenated furanones. Furanones occur at the surface of the alga in concentrations which strongly inhibit settlement of epibiota in laboratory and field assays, and are good examples of ecologically realistic "natural antifoulants". However, many other chemically rich seaweeds do not appear to use natural products as antifoulants, either because the compounds are not present at the surface of the plant, or because they do not persist at or near the surface in high enough concentrations to deter epibiota (polar metabolites in particular). We suggest that natural antifoulants in seaweeds will be most common for plants which produce non-polar metabolites and have morphologies which allow appropriate release of the metabolites. In contrast to this, polar metabolites may be more common as inducers of settlement for invertebrate larvae, and in this context we describe our recent work on induction of metamorphosis of larvae of the echinoid *Holopneustes purpurascens* by the floridoside - isethionic acid complex from red algae. Finally, it is now clear that bacteria use intricate chemical signalling sytems to regulate colonisation of surfaces and subsequent biofilm formation. Aspects of these bacterial studies will be contrasted with results from eukaryotes.

Antifouling activity of marine bacteria associated with seaweed surfaces.

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In an environment where all surfaces are constantly exposed to the threat of surface colonisation by fouling organisms many sessile marine organisms remain relatively free of biofouling. The ubiquitous nature of bacteria in the marine environment and their prominence during the early stages of biofilm development allows them to strongly influence the establishment of other fouling organisms. Over 400 strains of surface associated marine bacteria have been isolated from a range of marine organisms, primarily marine algae, using traditional culture media and media developed in our laboratory which better reflect the nutrients available in the natural environment. A large proportion of the isolated strains have been shown to inhibit the growth of and induce negative chemotaxis in fouling bacteria, isolated from a range of fouled surfaces. The regulation of antifoulant production by chemical signaling and environmental parameters has been demonstrated with extracts of potentially competing bacteria and algal extracts enhancing or inducing antifoulant production. Chemical fractionation of culture supernatants have yielded metabolites important in biofouling control with phenazine-1-ol and phenazine-1-carboxylic acid exhibiting potent antifouling activity. A third metabolite, the cyclic dipeptide Gly-Pro, appears to play a role in antifoulant production.

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Antifouling steroids against larval settlement of barnacles from marine sponge and octocorals

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Sessile marine invertebrates possess various chemical defense systems against predators, larval settlement of other fouling organisms, and pathogenic microbs. Their secondary metabolites, therefore, are potential nontoxic antifouling agents. We used cyprid larvae of the barnacle *Balanus amphitrite*, one of the most common fouling organisms, for screening of antifouling agents. During our search for antifouling substances from Japanese marine invertebrates, we found that the extracts of octocorals of the genus *Dendronephthya* (order Alcyonacea) and the marine sponge *Acanthella cavernosa* strongly inhibited cyprid settlement.

Bioassay-guided isolation from an octocoral *Dendronephthya* sp. afforded four D-*seco*steroids which showed potent antifouling activity (EC_{50} 2.2 mg/L). Cyprids treated with these D-*seco*steroids continued to swim without attaching to substrates for 7 days. The D-*seco*steroids were not lethal to the cyprid larvae even at 100 mg/L, which is much less toxic than $CuSO_4$. These results suggested that these D-*seco*steroids are promising for nontoxic antifoulants.

Since isogosterones A-D possess the unique D-*seco*steroid skeleton as well as considerable antifouling activity, we further examined extracts of several species of octocorals. From two octocorals *Dendronephthya* sp. and *Alcyonium gracillimum*, four new steroids possessing conjugated A-ring were isolated. These compounds were lethal to cyprids at 100 mg/L (LD_{100}), but they did not inhibit larval settlement at lower concentrations. Comparison of these steroids with the D-*seco*steroids, which had the same cross-conjugated A-ring, has revealed that a hemiacetal or an acetal moiety in steroidal skeleton may be crucial for antifouling activity, but (cross-)conjugated or aromatic A-ring were not important for the activity.

Four steroidal peroxides were isolated from the marine sponge *Acanthella cavernosa* as antifoulants. Interestingly, these compounds delayed larval settlement for 2-3 days when compared with the control experiments. The mode of action of these steroidal peroxides is of interest.

Incorporation of marine bacterial natural products into artificial surfaces and characterisation of their antifouling activity.

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Awareness of the harmful environmental effects of currently available antifouling paints and increasing legislation against their use has led to great interest in the way that marine organisms keep their surfaces clean and free from fouling. In this laboratory, we are investigating bacteria from seaweed and invertebrate surfaces. These bacteria appear to be able to produce secondary metabolites that reduce competition for space on surfaces. This release of compounds may benefit the macroalgae or invertebrate by reducing settling on its surfaces. Three bacterial strains showing promising antifouling activity were studied. Cells were harvested from cultures and extracted three times with methanol. The extracts were combined and evaporated to dryness. The supernatants from these cultures were run through an XAD column and eluted with either acetonitrile or methanol. Acetonitrile eluants were evaporated to remove the acetonitrile before being extracted with dichloromethane and evaporated. All of these extracts have been immobilised onto metal surfaces and remained active against other strains of fouling bacteria. This activity indicates that these natural products could be used as environmentally friendly antifouling compounds.

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Mearns-Spragg, A., Bregu, M., Boyd, K. G. & Burgess, J. G. (1998). Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Letters in Applied Microbiology* **27**, 142-146.

Evolutionary consequences of natural antifouling strategies: from bacteria to humans

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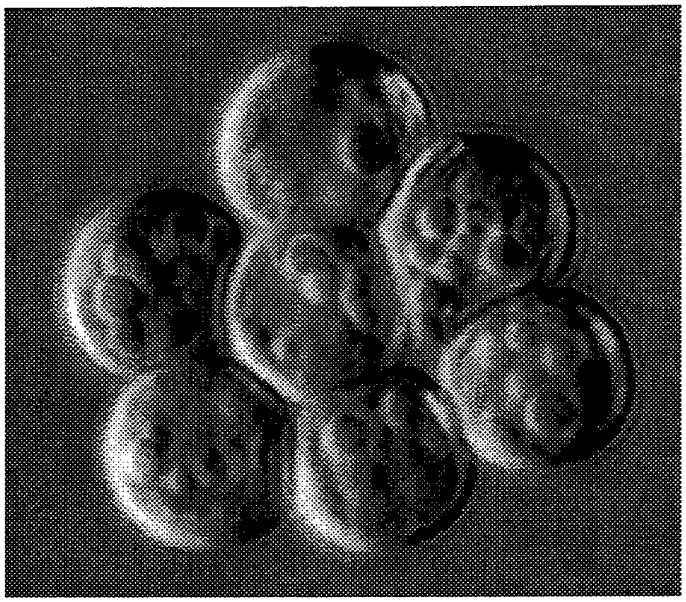
All organisms must have mechanisms for regulating adhesive interactions on their surfaces and these would have evolved very early in the history of life. Organisms must be able to avoid unwelcome adhesive interactions (e.g. immobilisation, predation, fouling) while facilitating desirable adhesive interactions (e.g. colonisation, feeding, locomotion). Most organisms have a outermost hydrophilic coating of sugar-rich molecules (glycocalyces) which are found from bacteria to vertebrates. This layer may be a default, low- adhesive surface, preventing unwanted non-specific adhesion by a combination of reducing hydrophobic adhesive interactions on the surface, providing a diffuse phase change at the surface and by localised ordering of the surrounding water molecules. From this came the evolution of extracellular matrices which allow the development of complex multicellular life. To adhere to these low adhesion surfaces has required the evolution of specific adhesive molecules and strategies, many of which are targeted at specific components of glycocalyces. This has led to an exquisite interplay between adhesive molecules and recognition sites and the need to distinguish between desirable and undesirable specific adhesion at surfaces. Organisms release sugar-rich secretions to counter specifically targeted but unwanted adhesive interactions. These outcompete the target sites on the glycocalyces for the adhesive molecules, thus preventing adhesion. This mechanism has led to muco-ciliary feeding systems and is extremely important in understanding biomedical questions of cell signalling and trafficking.

Abstracts



Poster Presentations

(1) Chemical cues for *Enteromorpha* settlement

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Kinetic analyses of settlement of zoospores of the green fouling alga *Enteromorpha*, show cooperativity. Spatial patterns suggesting gregarious settlement (autoaggregation) can also be detected. Gregarious settlement may be due to the release of chemical cues from previously settled spores or components of the microbial biofilm. It has been shown that surfaces coated with specific fatty acids cause an increase in the number of spores settling and the level of gregarious settlement. This effect appears to be chemoattractive in nature. This poster reports the results of experiments to identify natural chemical cues from *Enteromorpha* thallus and previously settled spores.

(2) The effect of cypris age on barnacle settlement: temporal nature of cypris perception of SIPC

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The settlement-inducing protein complex, SIPC, of the adult barnacle, *Balanus amphitrite* is a glycoprotein complex with lentil lectin (LCA) binding sites, and induces settlement of cyprids if adsorbed to substrata.

The response of cyprids to SIPC is influenced by a number of factors including cypris age. Relations between cypris age and response to SIPC were examined using a nitrocellulose membrane assay. Newly moulted cyprids were incubated at 25 °C in a cage of nylon plankton netting, and the settlement assay was performed at set periods. The results showed that relatively young cyprids tend to settle on SIPC-treated surfaces but aged cyprids can settle on surfaces even without SIPC. Interestingly, for the first few hours following the moult from the final nauplius stage, the cypris larvae appear unable to settle.

These results indicate that the ability of cyprids to settle is acquired during early ageing and that the ability to discriminate between SIPC-treated and untreated surfaces decreases with age. The changes in discriminatory ability during ageing are thought to be advantageous for 'gregarious' and 'pioneering' settlement strategies.

(3) Supply and settlement dynamics of *Semibalanus balanoides* cyprids: the weak link in the chain?

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Previous studies have examined the link between larval supply and settlement, or recruitment, for a variety of species at a range of scales. These studies have provided a number of useful insights into the deterministic and stochastic processes involved in benthic-pelagic coupling. Within this framework, this study aimed to examine, at the highest possible spatial and temporal resolution that was logistically possible, the supply and settlement of cyprids of the barnacle, *Semibalanus balanoides*. We constructed a floating frame that held 5 rows of 10 10x10 cm textured settlement panels manufactured from polyester resin. In the middle of each row a plankton pump provided continuous plankton samples to the surface. At the ends of each row were passive larval traps. Planktonic and settled cyprids were enumerated every low tide for 3 tidal cycles, then the tiles were replaced and the experiment repeated 2 more times. Current velocity and direction were also quantified at the site. The data show clearly the spatial and temporal variation in larval supply and settlement, however the relationship between the two depended on the resolution of the analysis. We found that if the data were aggregated then linear regression between mean number of cyprids per litre explained 78% of the variation in mean larval settlement (mean cyprids settled = 31.35 + 49.83 mean cyprids l^{-1}, $R^2 = 0.78$, $F = 21.85$, $P<0.05$). However if the data were analysed at the resolution as collected (i.e. by tide and depth) then only 25% of the variation in settlement was explained by supply (mean cyprids settled = 54.83 + 29.05 mean cyprids l^{-1}, $R^2 = 0.25$, $F = 12.86$, $P<0.05$). Peak settlement occurred at 0.8-1.0m below sea surface. Fixed arrays of tiles deployed at high, mid and low water demonstrated that the peak settlement zone shifted towards chart datum. The broad scale patterns observed on a rocky shore therefore seem not to be determined by larval supply.

(4) The timing of field settlement assays: the consequences of small differences in deployment date

Jeremy C. Thomason, J.M. Hills & P. Mapson

Department of Marine Science and Coastal Management, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK

One consequence of studying temperate marine ecosystems is a need to understand the effect of seasonal variation upon our data. Large scale variation, such as winter/summer, is readily observed and understood, however small scale temporal variation is often understated. In this study we made repeated deployments of tiles during the 6 week settlement period of the barnacle *Semibalanus balanoides*. Eight months later the tiles were examined and the density, morphology, fecundity and mortality of the adults were determined. This study show that relatively small shifts in the deployment of the settlement tiles affected colony density, body weight, body morphology, mortality but not fecundity. These results highlight the need for a thorough understanding of seasonal patterns in marine ecology and demonstrate the importance of repeated designs of fouling trials.

(5) The effect of hydrodynamics on the three-dimensional structure of barnacle colonies

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The shell morphology of barnacles can vary widely from short, squat individuals to tall, thin "trumpet" shaped individuals and very high densities of barnacles can lead to the formation of hummocks. This study aimed to determine the effect of flow regime on the three-dimensional structure of barnacle colonies in the field, to establish a link between hummock topography and shell orientation and to determine if there were dietary differences between individuals from hummocks and troughs.
Semibalanus balanoides larvae were allowed to settle in April 1997 on panels deployed facing either upstream, or downstream, at a site in the Clyde Sea (UK) with a predominately uni-directional flow. In October 1997 the panels were harvested and the density of barnacles, the three dimensional structure of the colonies and hummock characteristics were recorded for upstream and downstream facing colonies. Flow direction did not have a significant impact on the three-dimensional morphology. Carina-rostral orientation was not related to surface topography. Pyrolosis Mass Spectrometry was used to give a chemical finger print of individuals from hummocks and troughs on the upstream and downstream panels. Multiple Discriminant Analysis distinguished between these four groups suggesting that hydrodynamics and position in colony affect diet.

(6) Settlement behaviour of barnacle larvae in response to wood treated with a leaching-resistant anti-borer treatment containing copper, chromium and arsenic (CCA)

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At a range of European field sites, settlement intensity of barnacles on wood was less on untreated panels than on panels impregnated with CCA (a leaching-resistant anti-borer treatment containing copper, chromium and arsenic). Laboratory experiments were conducted to reveal the factors causing this phenomenon. The modification of surface chemistry has been investigated by measuring contact angles of drops of water and bubbles with respectively, dry and wet surfaces of treated and untreated wood. Settlement behaviour in response to treated and untreated surfaces was investigated under laboratory conditions. The effects of treatment on the surface microbial community developing during the period of the settlement experiments have been investigated by SEM. The relative importance of surface chemistry and microbial colonisation in determining settlement behaviour is discussed.

(7) Isolated fractions from the marine sponge *Geodia barretti* with inhibition of settlement in the barnacle *Balanus improvisus*

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The marine sponge *Geodia barretti* is almost entirely free of epibionts on its body surface. Based on this observation and the recent suggestions that peptides may play a role in marine communication we examined if high molecular weight fractions (1000-10000 Da) of a 50% ethanol extract occuring in *G.barretti* could provide biofouling protection. Two fractions were isolated from the sponge and tested in a laboratory bioassay based on settlement of *Balanus improvisus* larvae. Barnacle cyprids, obtained from laboratory cultures, were exposed for eight days to three fraction concentrations (10^{-3}, 10^{-4}, 10^{-5} mg ml^{-1}) in petri dishes. We found a complete inhibition of cyprid settlement in all concentrations and with no significant mortality. The presence and function of peptide receptors for external cues in barnacles is discussed and the high activity and low mortality of the tested high molecular weight fractions from *G.barretti* suggest a potential as an active component in future antifouling coatings.

(8) The antifouling benefits of a scallop / sponge symbiosis

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The scallop *Aequipecten* (*Chlamys*) *opercularis* is often found in association with the encrusting sponge *Suberites rubrus* The benefits of this association to either partner from this association have been demonstrated previously to be related to enhance predator avoidance. In this study we show that the sponge also gains positive benefit from the antifouling activity of the sponge. Field trials show that the sponge prevents settlement of cypris larvae and laboratory studies show the increase in hydrodynamic drag associated with a fouling burden. Fouling of shells of commercially produced scallops is a recognised problem and it is suggested that *Suberites* may make a useful, natural and acceptable antifouling coating.

(9) Non-toxic defence against fouling with natural and synthetic repellents

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Many commonly used methods of protecting ship hulls, oil platforms, pipelines, etc. against fouling are based on the use of toxic compounds which contaminate the environment. At present, only few non- toxic antifoulants have been found. It was shown that benzoic and tannin acids, *N,N,N`,N`*-tetramethylethylenediamine, acrilamide repelled marine bacteria (Chet, Mitchell, 1976). We hypothesise that non-toxic defence against biofouling might be realised by suppression of larval settlement through repellents. In this study we investigated the effect of some synthetic and natural repellents on marine fouling. In the White Sea, we found that some algae (e.g. *Laminaria saccharina*) reduced fouling on their surfaces and on artificial substrata up to several *dm* away from it. These reduction of larval settlement was a result of excretion of repellents from this alga. Field experiments with extracts of *L.saccharina* incorporated into non-toxic matrix (Phytogel) showed that the fouling on experimental plates was reduced 3 times compared to untreated controls. Synthetic repellents (tetramethylethylenediamine, benzoic acid) in the non-toxic concentrations which were included in the varnish defended against both micro- and macrofouling as well as a ship-hull paint (KhV-5351, Pigment Co) Laboratory experiments with capillaries and chemotactic chambers showed that these substances were non-lethal for foulers but repelled larvae of *Mytilus edulis*, and hydroids *Obelia loveni* and *Dynamena pumila*. Our study showed that some algae and synthetic repellents reduced or inhibited fouling effectively by preventing active settlement of larvae but they did not kill them. On the base of this approach a non-biocidal defence against fouling could be developed.

(10) Electrochemical method of protection against marine growth adhering to ship hulls

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The developed electrically driven method of protection against marine growth is based on the principle that the pH-value is continuously changed in a very thin (µm range) water film on the surface of a submerged object to be protected (ship shell plating, offshore structure) by a periodical electrical drive of the conducting outer skin of this surface causing that neither larvae of macroorganisms, nor bacteria or diatoms (primary mucilage) can adhere to it or establish themselves

To this end it is not intended to achieve an extreme acidity or extreme alacaline level but a permanent change in the ph values. Experience has shown that often changes in the abiotic factors have had a negative influence on the growth of organisms.

The a.m. principle is technically applied through a special electrical drive system as well as a three-layer ground coat system consisting of:
a priming coat which ensures anti-corrosion protection and electrical insulation to the ship´s hull,
a thin titanium coating which is electrically contacted and provides for charge distribution over the entire surface to be protected, and
an electrically conductive electrolysis- and seawater-resistant polymer external coating on which the pH-value change itself occurs depending on electrolysis and which protects the titanium coating under it from passivation and damage.
Up to now these are results from panel-experiments. The next step is application of the system to a ship.

Information about economic considerations and compatibility with the environment are also given at the poster.

(11) Biofouling of optical instruments: The problem and a potential solution.

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Optical instruments are now widely used in oceanography and the offshore industries to measure environmental flux and for imaging in the marine environment. During prolonged deployment times the instruments are susceptible to problems associated with biofouling. Where a fouling assemblage is able to accumulate on the sensor windows of an optical instrument, the optical signal through the window can be disrupted, compromising the integrity of the measurements being recorded.

Both the microbial nature of the biofouling, and the requirements for maintaining high optical quality at the surfaces of the window make traditional coating based approaches to antifouling inappropriate for many optical sensors. The relatively small size of optical sensor windows allows some antifouling technologies that are usually limited by costs or range to be considered including sonication, electrodes and ultra violet radiation (UVR).

Here we report that fouling biofilms rapidly developed on glass surfaces, and the viewing ports of underwater video cameras, deployed at shallow sites in the Largs Channel, Scotland. Studies using scanning spectrophotometry showed this accumulation of biofilm reduced the transmission of light through the fouled slides, across a broad spectrum of wavelengths (350-850nm). The video images also showed considerable deterioration as the biofouling accumulated.

During this study a range of antifouling technologies have been developed and tested to protect optical windows. The results from some successful laboratory and sea trials will be discussed and some limitations to the technologies discussed.

(12) Screening of some marine organisms from Brittany's shores (France) for potential antifouling activities.

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The control of biofouling is of particular concern to modern marine engineering and shipping operations. Current antifouling technologies rely largely on heavy metal-based paints which act as broad spectrum toxins due to the controlled release of copper or tin compounds. Because of the significant environmental damage caused by this activity on non-target organisms, the use of such paints is restricted (de Nys *et al.*, 1995). Many marine organisms appear relatively free from fouling compared with man made submerged structures and furthermore the inhibition of fouling is usually observed among living organisms. Studies on antifouling mechanisms of marine organisms may provide valuable information for fouling control in marine technology and can be useful for the search of new compounds for marine coatings more safety for the environment. In this study the antifouling activity was investigated on a series of aqueous, ethanolic and dichloromethane extracts from 15 algae from Brittany's shores (North West of France). The extracts were tested in laboratory assays against representatives organisms involved in the fouling process :

Primary film :

Bacteria gram- (*E. Coli K12, Klebsiella pneumoniae, Serratia mercescens, Proteus vulgaris, Pseudomonas aeruginosa*), **bacteria gram+** (*Bacillus subtilis, B. cereus, B. megaterium, Staphyloccocus aureus, Streptococcus* sp.), **the yeasts** (*Issatchenkia orientalis, Candida tropicalis, , C. albicans, C. brusei , Saccharomyces cerevisiae*), **the marine fungi** (*Corollospora maritima, Lulworthia sp., Dendryphiella salina*),

Secondary film :

the diatoms (*Amphora coeffeaformis, Cylindrotheca closterium* and *Phaeodactylumtricornutum*), **the spores of macroalgae** (*Ulva lactuca, Sargassum muticum* and *Enteromorpha sp.*)

Tertiary film :

the blue **mussels** *Mytilus edulis*

The extracts tested, as a group, exhibited broad spectrum activity. Moreover, several individual compounds were also relatively broad spectrum by themselves, showing moderate to high levels of activity in all assays. No single compound was the most active extract in all tests. The variation in activity suggest that particular metabolites may be able to be used for specific situations thus minimizing effects on non-target organisms. Further investigation of purification of the two more active extract are actually in progress.

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